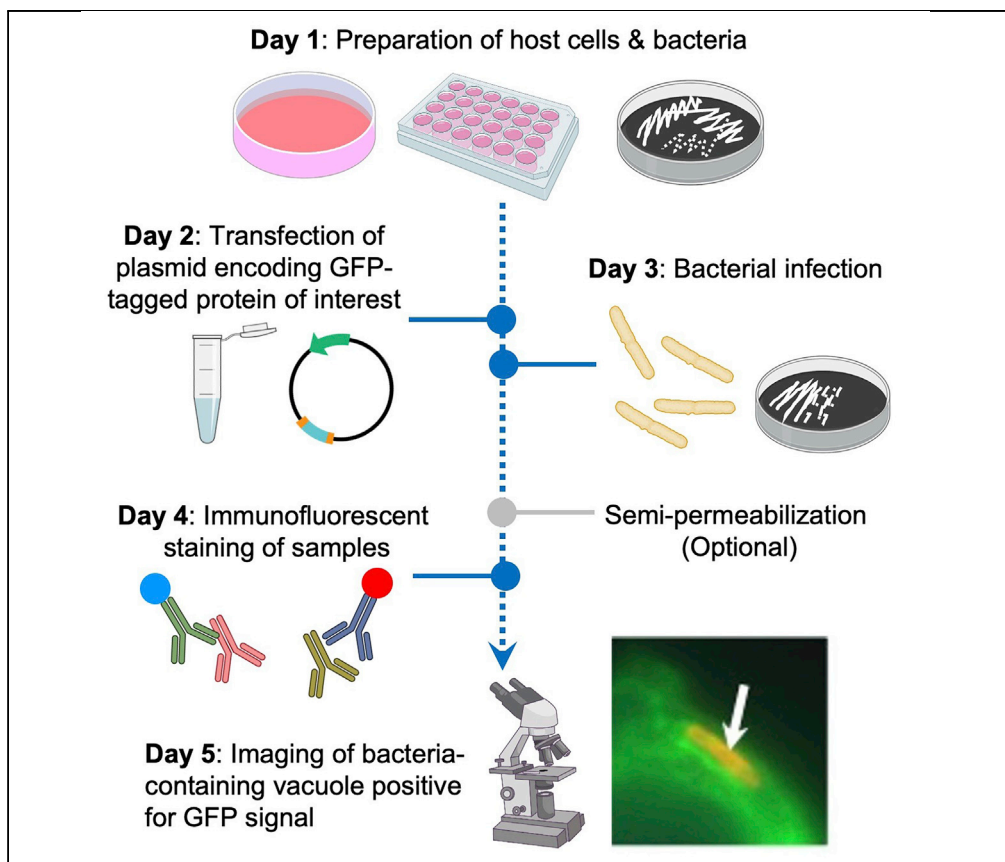


## Protocol

# Protocol for imaging proteins associated with *Legionella*-containing vacuoles in host cells



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### Highlights

Step-by-step workflow describing how to infect host cells with intracellular bacteria

Identification of subcellular localization of bacterial proteins in host cells

Analysis of cellular reorganization of host proteins as a result of infection

Protocol is applicable to any intracellular bacteria

The intracellular bacterial pathogen *Legionella pneumophila* exploits host cellular systems using approximately 300 effector proteins to establish a replicative niche known as the *Legionella*-containing vacuole (LCV). During infection, both host and bacterial proteins interactively function on the LCVs. Here, we describe a detailed step-by-step protocol to visualize proteins associated with LCVs in host cells. This protocol can aid in analyzing whether a protein of interest influences the subcellular localization of LCV-associated proteins during infection.

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## Protocol

Protocol for imaging proteins associated with *Legionella*-containing vacuoles in host cellsTomoe Kitao,<sup>1,4,5,\*</sup> Kohei Arasaki,<sup>3</sup> Hiroki Nagai,<sup>1,2,\*</sup> and Tomoko Kubori<sup>1,2,\*</sup><sup>1</sup>Department of Microbiology, Graduate School of Medicine, Gifu University, Gifu, Gifu 501-1194, Japan<sup>2</sup>G-CHAIN, Gifu University, Gifu, Gifu 501-1194, Japan<sup>3</sup>School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [kitao@gifu-u.ac.jp](mailto:kitao@gifu-u.ac.jp) (T.K.), [hnagai@gifu-u.ac.jp](mailto:hnagai@gifu-u.ac.jp) (H.N.), [tkubori@gifu-u.ac.jp](mailto:tkubori@gifu-u.ac.jp) (T.K.)  
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## SUMMARY

The intracellular bacterial pathogen *Legionella pneumophila* exploits host cellular systems using approximately 300 effector proteins to establish a replicative niche known as the *Legionella*-containing vacuole (LCV). During infection, both host and bacterial proteins interactively function on the LCVs. Here, we describe a detailed step-by-step protocol to visualize proteins associated with LCVs in host cells. This protocol can aid in analyzing whether a protein of interest influences the subcellular localization of LCV-associated proteins during infection.

For complete details on the use and execution of this protocol, please refer to Kitao et al. (2020).

## BEFORE YOU BEGIN

In this manuscript, we describe a step-by-step protocol of immunofluorescence microscopy to analyze whether a target protein can localize to a bacteria-containing vacuole during infection using bacteria-infected cells. In addition, we describe an optional protocol to examine whether a protein of interest influences the subcellular localization of the bacterial vacuole-associated proteins during infection by using a semi-permeabilization technique (Kitao et al., 2020; Kubori et al., 2017).

We use the intracellular bacterial pathogen *Legionella pneumophila* as a model organism for this protocol. According to biosafety guidelines, *L. pneumophila* is classified as a biosafety level 2 organism. Any experiments using *L. pneumophila* described here should be approved by the institutional biosafety committee and carried out according to the recombinant DNA committee guidance. Once approved, please make sure that the reagents and materials shown below are ready for use before starting the main protocol.

## Preparation of bacterial strain(s)

⌚ Timing: 3 days

When analyzing whether a *L. pneumophila* effector protein of interest influences the subcellular localization of host protein that is already known to be recruited to the *Legionella*-containing vacuole (LCV) upon infection, use the *L. pneumophila* wild-type strain (positive control) and its isogenic mutant(s) lacking the gene(s) encoding the effector protein(s).



Prepare the *L. pneumophila* deletion mutant strains as per the protocol described previously (Zuckman et al., 1999).

1. Pick up a trace of the *L. pneumophila* wild-type strain Lp01 and its isogenic mutant(s) lacking the gene(s) of interest, from the stocks stored at  $-80^{\circ}\text{C}$ , using autoclaved flat toothpicks, and streak them on charcoal yeast extract (CYE) agar plates.
2. Incubate the plates at  $37^{\circ}\text{C}$  for 72 h (single colonies usually appear after 3 days).

### Maintenance of host cells

⌚ Timing: 3 days

We use exponentially growing healthy HEK293T cells that constitutively express Fc gamma receptor II (HEK293T-Fc $\gamma$ RII) or HeLa-Fc $\gamma$ RII cells in this protocol for *Legionella* infection experiments. Both cell lines were maintained in 10-cm cell culture dishes containing 10 mL Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 200  $\mu\text{g}/\text{mL}$  hygromycin.

3. Thaw HEK293T-Fc $\gamma$ RII or HeLa-Fc $\gamma$ RII cells from the liquid nitrogen stocks and transfer them into a 15-mL conical tube containing 10 mL prewarmed DMEM supplemented with 10% FBS.
4. Centrifuge the tube with swing buckets at  $220 \times g$  for 5 min at room temperature ( $15^{\circ}\text{C}$ – $25^{\circ}\text{C}$ ).
5. Remove the supernatant and resuspend the precipitated cells in 10 mL of prewarmed DMEM supplemented with 10% FBS.
6. Plate the cell suspension into 10-cm cell culture dish.
7. Incubate at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 24 h.
8. Detach the cells from the dish.
  - a. HEK293T-Fc $\gamma$ RII.
    - i. Remove the cell culture medium from a dish with semi-confluent cells.
    - ii. Add 10 mL of prewarmed fresh DMEM supplemented with 10% FBS.
    - iii. Suspend the surface-attached cells by pipetting (detach by mechanical force).
  - b. HeLa-Fc $\gamma$ RII.
    - i. Remove the cell culture medium from a dish with semi-confluent cells.
    - ii. Add 2 mL of trypsin/EDTA solution to the dish. (This treatment induces chemical detachment of the cells from the surface of the dish).
    - iii. Incubate the dish at  $37^{\circ}\text{C}$  for 5 min in a  $\text{CO}_2$  incubator.
    - iv. Gently tap the dish to completely detach the cells.
    - v. Add 8 mL of prewarmed fresh DMEM supplemented with 10% FBS and suspend the cells by gentle pipetting.
9. Cell passage: transfer the detached cells with an appropriate dilution to a new dish.
  - a. HEK293T-Fc $\gamma$ RII: Transfer 1 mL of the cell suspension to a new dish containing 9 mL of prewarmed DMEM supplemented with 10% FBS (1:10 split).
  - b. HeLa-Fc $\gamma$ RII: Transfer 2 mL of the cell suspension to a new dish containing 8 mL of prewarmed DMEM supplemented with 10% FBS (1:5 split).
10. Add 40  $\mu\text{L}$  of 50 mg/mL hygromycin.

**⚠ CRITICAL: Hygromycin is required for stable expression of Fc $\gamma$ RII in our cell lines. We added hygromycin from the second passage.**

11. Incubate the dishes containing the diluted cells at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in an incubator for 3 days to allow them to reach confluency.

### Plasmid preparation for transfection

⌚ Timing: 24–36 h

We used endotoxin-free plasmids for transfection. Endotoxin-free plasmids can be prepared using EndoFree Plasmid MAXI prep kits (QIAGEN) according to the manufacturer's instructions. This protocol describes how to detect GFP-tagged proteins of interest recruited to bacterial vacuoles during infection. A plasmid encoding a GFP-tagged protein must be constructed before starting this protocol. A plasmid encoding only GFP is required as a negative control.

12. Pick up a trace of *Escherichia coli* DH5 $\alpha$  carrying a plasmid encoding GFP or GFP-tagged protein from  $-80^{\circ}\text{C}$  frozen stocks using a sterilized flat toothpick and streak them on LB agar plates containing 20  $\mu\text{g}/\text{mL}$  kanamycin. (The vector used in this protocol encodes a  $\beta$ -lactamase gene that confers kanamycin resistance).
13. Incubate the plate at  $37^{\circ}\text{C}$  overnight (18–20 h).
14. Pick up a single colony and inoculate them in 100 mL LB medium containing 20  $\mu\text{g}/\text{mL}$  kanamycin in a 200 mL flask.
15. Shake at  $37^{\circ}\text{C}$  and 250 rpm, overnight (16–18 h).
16. Transfer the bacterial culture into a 50-mL conical tube and centrifuge at  $6,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ .
17. Remove the supernatant.

▮▮ **Pause point:** The bacterial pellets can be stored at  $-80^{\circ}\text{C}$  until purification.

18. Purify the plasmid according to the manufacturer's instructions.

### Purified protein

If you have a purified protein of interest that may influence the LCV-associated proteins, you can test the protein by using semi-permeabilized host cells infected with *L. pneumophila*. Prepare 100–200  $\mu\text{L}$  of 1–2 mg/mL protein if necessary.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti- <i>Legionella</i> rat antibody	Scrum	Cat# 17457
Anti- <i>Legionella</i> rabbit antibody	BioAcademia	Cat# 64-100
Alexa Fluor 350-conjugated anti-rat IgG	Thermo Fisher Scientific	Cat# A21093; RRID:AB_10563602
Rhodamine Red-X-conjugated anti-rabbit IgG	Thermo Fisher Scientific	Cat# R6394; RRID:AB_2556551
<b>Bacterial strains</b>		
<i>Legionella pneumophila</i> Philadelphia-1 (Lp01)	Berger and Isberg, 1993	NC_002942.5
<i>Escherichia coli</i> DH5 $\alpha$	Toyobo	Cat# DNA-903
<b>Chemicals</b>		
ProLong™ Diamond Antifade Mountant	Thermo Fisher Scientific	Cat# P36961
DMEM (1 $\times$ )	Gibco	Cat# 11885-084
Opti-MEM	Gibco	Cat# 31985-062
Goat serum	Gibco	Cat# 16210-064
Bacto™ agar	BD	Cat# 214010
Bacto™ yeast extract	BD	Cat# 212750
Activated charcoal	Sigma	Cat# C5510-500G
FBS	Sigma	Cat# 172012-500ML
PBS	Sigma	Cat# D8537-500ML
PFA	Sigma	Cat# 441244-1KG
ACES	Sigma	Cat# A3594-1KG
Activated charcoal	Sigma	Cat# C5510-500G
L-Cysteine hydrochloride monohydrate	Nacalai Tesque	Cat# 10313-55

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kanamycin sulfate	Nacalai Tesque	Cat# 19860-31
Iron(III) nitrate enneahydrate	Nacalai Tesque	Cat# 19514-55
Trypsin/EDTA solution	Nacalai Tesque	Cat# 32777-44
Hygromycin	Nacalai Tesque	Cat# 09287-84
Triton X-100	Nacalai Tesque	Cat# 35501-15
Polyethyleneimine (PEI)	Polysciences	Cat# 24765-2
Poly-L-lysine (PLL)	Millipore	Cat# A-005-C
<b>Critical commercial assays</b>		
EndoFree Plasmid MAXI prep kits	QIAGEN	Cat# 12362
<b>Experimental models: cell lines</b>		
Human: HEK293T-FcγRII	<a href="#">Arasaki and Roy, 2010</a>	N/A
Human: HeLa-FcγRII	<a href="#">Arasaki et al., 2017</a>	N/A
<b>Experimental models: organisms/strains</b>		
<i>Legionella pneumophila</i> Philadelphia-1 (Lp01)	Berger and Isberg, 1993	NC_002942.5
<b>Recombinant DNA</b>		
PmGFP	<a href="#">Kitao et al., 2020</a>	N/A
<b>Software and algorithms</b>		
NIS-Elements AR 3.2 64-bit	Nikon	<a href="https://www.nsl.nikon.com/">https://www.nsl.nikon.com/</a>
<b>Others</b>		
Cell culture 24-well plate	TrueLine	Cat# TR5002
10 cm Cell culture dish	TrueLine	Cat# TR4002
10 mL Conical tube	TrueLine	Cat# TR2001
0.22 μm Disk filter (Millex-GP)	Millipore	Cat# SLGPR33RB
1.5 mL Microfuge tube	Eppendorf	Cat #0030125150
Sterilized petri dish	AS ONE Corporation	Cat# 6-9742-01
Round coverslips	Fisher Scientific	Cat# 12-545-80
Sterilized swabs (2.6 × 11 × 150 mm)	Kawamoto	Cat# 023-412010-00
Slide glasses	Matsunami	Cat# S1126

## MATERIALS AND EQUIPMENT

### Preparation of bacterial strains

- LB agar plates containing 20 μg/mL kanamycin
- Sterilized swabs
- Flat toothpicks
- CYE plates

Dissolve ACES and yeast extract in approximately 800 mL of MilliQ water, then adjust the pH to 7.0 with KOH. After adjusting the volume to 1,000 mL, add it into a 2-L-flask containing charcoal and bacto agar. Autoclave it at 121°C for 20 min. After autoclaving, allow it to cool to around 60°C in

Reagent	Final concentration	Amount
ACES	1%	10 g
Yeast extract	1%	10 g
MilliQ	-	Up to 1000 mL
Activated charcoal	0.2%	2 g
Bacto Agar	1.5%	15 g

a water bath. Prepare 40 mg/mL of L-cysteine solution (>10 mL) and 13.5 mg/mL of Iron(III) nitrate solution (>10 mL) separately in sterilized MilliQ water and filter them with a 0.22  $\mu\text{m}$  disk filter. Add 10 mL of each to 1 L autoclaved CYE (The L-cysteine should be added first, followed by the iron solution to prevent precipitation.). If needed, add appropriate concentrations of antibiotics. Mix well using a magnetic stirrer and pour the media into sterilized petri dishes (approximately 30–40 mL/dish). Let the media cool down and dry the plates 2 overnights at room temperature (15°C–25°C). The CYE plates can be stored at 4°C. Remove moisture on the lids before the plates are stored.

- Spectrophotometer
- Disposable cuvette

### Host cell maintenance

- Cell culture-treated dishes (10-cm)
- Cell culture-treated plates (24-well)
- DMEM supplemented with 10% FBS filtered with 0.22  $\mu\text{m}$  filter
- Hygromycin (50 mg/mL)
- Trypsin/EDTA solution
- Centrifuge for 24-well plates
- Water bath
- 37°C CO<sub>2</sub> incubator

### Transfection

- Polyethyleneimine solution (PEI)

Dissolve PEI at a concentration of 2.0 mg/mL in MilliQ water, adjust the pH to 8.0 with 1N NaOH. Then, filter it with a 0.22  $\mu\text{m}$  disk filter. The solution can be dispensed into small aliquots (~1 mL) and stored at –20°C.

### Immunofluorescent staining

- 4% Paraformaldehyde (PFA)
- Phosphate buffer saline (PBS)
- 2% Goat serum diluted with PBS (2% GS/PBS)
- 0.2% Triton X-100
- Glass slides
- Sterilized round coverslips
- ProLong™ Diamond antifade mountant
- Semi-permeabilization buffer (Optional)

Dissolve glucose in 450 mL water and add all the remaining reagents. After making-up the volume to 500 mL using MilliQ water, filter it with Stericup (0.22  $\mu\text{m}$ ).

Reagents	Stock concentration	Amount	Final concentration
Hepes-KOH	1M	12.5 mL	25 mM
Potassium acetate	1M	62.5 mL	125 mM
Magnesium acetate	1M	1.25 mL	2.5 mM
Glucose	n/a	0.5 g	1 mg/mL
ddH <sub>2</sub> O	n/a	Up to 500 mL	n/a

DTT (final concentration 1 mM) is added before use.

## STEP-BY-STEP METHOD DETAILS

### Preparation of bacterial strains (day 1)

⌚ Timing: 30–45 min

For the infection experiment, on day 3, pick up a single colony of bacteria and re-streak on a new CYE agar plate to prepare “2-day-heavy patch” of the strain.

1. Warm up a new CYE plate at 37°C to remove condensation.
2. Pick up a single colony using a sterilized flat toothpick and re-streak it on the CYE plate.
3. Incubate the plate at 37°C for 2 days (48 h).

### Host cell preparation (day 1)

⌚ Timing: ~2 h

Before starting this protocol, please make sure that you have semi-confluent cell culture plates. A single semi-confluent plate provides a sufficient number of cells to prepare one 24 well plate. For microscopy, we seed the cells in 24-well cell culture plates with round glass coverslips 24 h before transfection. For the HEK293T-FcγRII cells, the coverslips should be coated with poly-L-lysine (PLL) as described in steps 4–7 to increase the adherence of the cells. For the HeLa-FcγRII cells, steps 5–7 can be skipped.

4. Place sterilized round glass coverslips in the 24-well cell culture plates. Set them up in triplicates for each sample. For example, if you analyze the subcellular localization of a GFP-tagged protein in host cells infected with two *L. pneumophila* strains, such as the wild-type and an isogenic mutant strain, you need to prepare the cells in six wells (three wells for the wild-type strain and three wells for the mutant strain).
5. Add 250 μL of 0.001% (w/v) poly L-lysine solution to the coverslip in each well of the 24-well plates. Sink the coverslips using a sterilized pipet if they begin to float.
6. Gently incubate the plates in an orbital shaker for 1 h at room temperature (15°C–25°C).
7. Remove the poly-L-lysine solution and wash the coverslips in the wells with 500 μL/well of sterilized MilliQ water three times.
8. Remove the cell culture medium from the semi-confluent 10 cm dish with cells.
9. Add prewarmed 10 mL PBS.
10. Detach the cells from the dish.
  - a. For HEK293T-FcγRII cells
    - i. Remove PBS.
    - ii. Add 10 mL of prewarmed fresh DMEM supplemented with 10% FBS.
    - iii. Detach the cells by pipetting.
    - iv. Transfer the cells to a sterilized 50-mL conical tube.
  - b. For HeLa-FcγRII cells
    - i. Remove PBS.
    - ii. Add 2 mL of the Trypsin/EDTA solution.
    - iii. Incubate the dish for 5–10 min in a 37°C CO<sub>2</sub> incubator.
    - iv. Gently tap the dish to detach the cells.
    - v. Add 8 mL of prewarmed fresh DMEM supplemented with 10% FBS and suspend the cells by gentle pipetting.
    - vi. Transfer the cells to a sterilized 50-mL conical tube.
11. Count the cell number using a hemocytometer.
12. Seed the cells into 24-well plates with PLL-coated coverslips ( $0.8 \times 10^5$  cells/well for HEK293T-FcγRII cells;  $0.4 \times 10^5$  cells/well for HeLa-FcγRII cells).

△ **CRITICAL:** Use hygromycin-free DMEM supplemented with 10% FBS.

13. Incubate the plate at 37°C in a CO<sub>2</sub> incubator for 24 h.

### Plasmid transfection (day 2)

⌚ **Timing:** 30–50 min

14. Replace the cell culture medium with fresh and prewarmed DMEM supplemented with 10% FBS.
15. Twenty-four hours prior to infection, transfect the host cells with plasmid DNA encoding a GFP-tagged desired protein using PEI. In the following protocol, the indicated amount of reagents is required for cells in a single well.
  - a. Dilute 1 μL of PEI with 50 μL of Opti-MEM (Gibco) in a 1.5-mL microfuge tube.
  - b. Incubate the tube for 15 min at room temperature (15°C–25°C).
  - c. Add 1.25 μL of a 100 ng/μL endotoxin-free plasmid.
  - d. Incubate the tube at room temperature (15°C–25°C) for 15 min.
  - e. Drop PEI/plasmid/Opti-MEM mixture into cells seeded in the 24-well plates.

**Alternatives:** We used PEI for transfection to prevent saturated signals of GFP-tagged protein. Other transfection reagents, such as lipofectamine, can also be used to transfect host cells. Follow the manufacturer's instructions for each product.

16. Incubate the plates at 37°C in a CO<sub>2</sub> incubator for 24 h.

### Legionella infection (day 3)

⌚ **Timing:** 2 h-1 day \*Varies with time of infection

The following protocol is an example of a case in which host cells are infected with *L. pneumophila* at a multiplicity of infection (MOI) of 5.

17. Prepare *L. pneumophila* suspension in sterilized MilliQ water.
  - a. Scrape the appropriate amount of a 2-day heavy patch of *L. pneumophila* grown on a CYE plate using a sterilized pipette tip (1,000 μL size) or flat toothpick and resuspend in 500 μL of sterilized MilliQ water.
  - b. Mix the bacterial solution well using a vortex and measure the absorbance of the suspension using a spectrophotometer at 600 nm (OD<sub>600</sub>).
  - c. Prepare the bacterial suspension with an OD<sub>600</sub> value of 0.1, which approximately corresponds to 1 × 10<sup>5</sup> bacteria/μL, in a new 1.5-mL microfuge tube.
18. Opsonize *L. pneumophila* with anti-*Legionella* antibody to enhance FcγRII-mediated bacterial internalization. The preparation shown below is sufficient for cells in three wells.
  - a. Prepare 2 mL of prewarmed DMEM supplemented with 10% FBS in a sterilized 10-mL conical tube.
  - b. Add 0.7 μL (1/3,000 volume) of anti-*Legionella* rabbit serum.
  - c. Add *L. pneumophila* (16 μL of 1 × 10<sup>5</sup> cells/μL *L. pneumophila* for HEK293TFcγRII; 8 μL of 1 × 10<sup>5</sup> cells/μL *L. pneumophila* for HeLa-FcγRII).
  - d. Shake the tubes at 37°C at 250 rpm for 30 min.
19. Infect cells with the opsonized *L. pneumophila*.
  - a. Remove the cell culture medium from the wells of the 24-well cell culture plates and dispense DMEM containing the opsonized *L. pneumophila* prepared in step 18 to the wells (500 μL/well).



- b. Centrifuge the cell culture plate at  $220 \times g$  for 5 min at room temperature ( $15^{\circ}\text{C}$ – $25^{\circ}\text{C}$ ) with a swing bucket rotor. This procedure involves the attachment of bacteria to the surface of the adherent cells because the *L. pneumophila* strains used here are non-motile.
  - c. To initiate infection, float the plate in a  $37^{\circ}\text{C}$  water bath for 5 min immediately after the centrifuge is stopped (Time zero of infection).
  - d. Incubate the plate at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator for 55 min.
20. At 1-h post-infection, gently wash the cells with prewarmed PBS (1 mL/well) three times to remove extracellular bacteria, and then add prewarmed DMEM supplemented with 10% FBS. Resume the incubation of the plate at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator (time can vary as per the requirement).

**Alternatives:** You can skip step 20 if the sample is taken at 1 h or less post infection.

21. Gently wash the cells with prewarmed PBS (1 mL/well) three times at the time of sampling.
  22. Remove PBS and add  $500 \mu\text{L}$ /well of 4% PFA/PBS solution to fix the cells and incubate the plates at room temperature ( $15^{\circ}\text{C}$ – $25^{\circ}\text{C}$ ) for 15 min.
23. Wash the cells with PBS (1 mL/well) three times.

**Optional:** The following protocol is to analyze whether a protein of interest may influence another protein, which is already known to be recruited to LCVs, by using the combination of a purified protein and semi-permeabilized host cells. Digitonin is used for semi-permeabilization in this protocol. At a given concentration of digitonin permeabilizes only plasma membrane (Arasaki et al., 2012). In some cases, the concentration of digitonin may be required to be optimized.

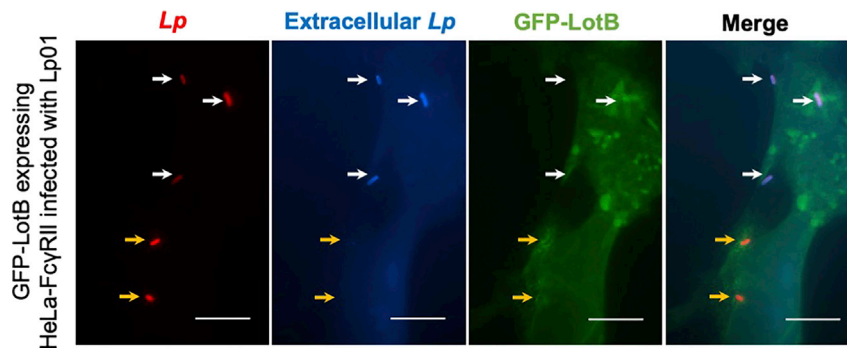
- a. After *L. pneumophila* infection, wash the cells on the coverslips in the 24-well cell culture plates with prewarmed  $500 \mu\text{L}$ /well of the semi-permeabilization buffer twice.
- b. Remove the semi-permeabilization buffer and add  $500 \mu\text{L}$ /well of fresh semipermeabilization buffer containing  $3 \mu\text{g}/\text{mL}$  digitonin.
- c. Incubate at room temperature ( $15^{\circ}\text{C}$ – $25^{\circ}\text{C}$ ) for 4 min.
- d. Gently wash the cells with  $500 \mu\text{L}$ /well of semi-permeabilization buffer three times.
- e. Add  $250 \mu\text{L}$ /well of semi-permeabilization buffer containing 1–10  $\mu\text{g}/\text{mL}$  protein to be analyzed. Pilot tests should be done to determine the optimal concentration of protein in advance.
- f. Incubate the plates at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator for 1 h to allow the protein to access to the cell cytosol where LCVs are located.
- g. Wash the cells with prewarmed  $500 \mu\text{L}$ /well of the semi-permeabilization buffer twice.
- h. Remove the buffer and add  $500 \mu\text{L}$  of 4% PFA/PBS solution and incubate the plates at room temperature ( $15^{\circ}\text{C}$ – $25^{\circ}\text{C}$ ) for 15 min to fix the cells.
- i. Wash the cells with PBS (1 mL/well) three times.

**Pause point:** You can pause the experiment here if needed. In this case, cover the plates with aluminum foil to protect them from light and evaporation and store them at  $4^{\circ}\text{C}$ .

### Immunofluorescent staining (day 4)

⌚ **Timing:** 1 day

When the infected cells are semi-permeabilized and treated with the protein as described in the previous section, begin the procedure from step 29. If not, begin from step 24. If the semi-permeabilization technique is not used, the extracellular bacteria should be immunologically stained with a blue signal to distinguish them from the intracellular bacteria (Figure 1). This procedure can help



**Figure 1. Example of extracellular uninfected bacteria staining**

HeLa-Fc $\gamma$ RIII cells transfected with plasmids encoding GFP-LotB and the plasmid encoding FLAG- $\beta'$ -COP were infected with Lp01 at an MOI of 5. After 4 h, the cells were fixed, immunostained and visualized using a fluorescence microscope. White and yellow arrows indicate extracellular and intracellular *L. pneumophila* cells, respectively. By using this protocol, all of *L. pneumophila* cells used to infect host cells can be stained with red fluorescence signal, whereas extracellular uninfected cells can be stained with both red and blue fluorescence signals. Scale bars, 10  $\mu$ m.

exclude the extracellular uninfected bacteria in the imaging analysis step and the LCV counting process.

24. Blocking: Add 500  $\mu$ L of 2% GS/PBS (2% goat serum prepared in PBS) per well to the fixed cells.
25. Incubate the plates at room temperature (15°C–25°C) for 30 min.
26. Remove the coverslips using sharp-tip tweezers from the wells of the plate and place them upside down on the drops (40  $\mu$ L) of diluted anti-*Legionella* rat serum (1:5,000 dilution) prepared in 2% GS/PBS to stain uninfected extracellular bacteria. (Set up reactions on the parafilm placed on the flat surface.)
27. Incubate the coverslips at room temperature (15°C–25°C) for 1 h in a closed container.
28. Replace the coverslips in the wells with 1 mL of PBS (cell-side up) in the 24-well plates. Wash the coverslip with PBS (1 mL/well) three times.
29. Remove PBS and add 500  $\mu$ L of 0.2% Triton X-100 in PBS to permeabilize the cell organelles so as to stain bacteria residing inside the LCV.
30. Gently rock the plates using an orbital shaker at room temperature (15°C–25°C) for 20 min.
31. Wash the cells with PBS (1 mL/well) three times.
32. Add 500  $\mu$ L of blocking solution (2% GS/PBS) per well.
33. Incubate the plates at room temperature (15°C–25°C) for 30 min.
34. Remove the coverslips from the wells of the plates and place them on drops (40  $\mu$ L) of diluted anti-*Legionella* rabbit serum (1:5,000 dilution) prepared in 2% GS/PBS to stain intracellular bacteria.
35. Incubate the coverslips at room temperature (15°C–25°C) for 1 h in a closed container.
36. Replace the coverslips in the wells with 1 mL of PBS (cell-side up) in the 24-well plates. Wash the coverslips with PBS (1 mL/well) three times.
37. Secondary antibody reaction.
  - a. Cells without semi-permeabilization.
    - i. Remove the coverslips from the wells of the plates and place them on drops (40  $\mu$ L) of the secondary antibody solution containing Alexa Fluor 350-conjugated anti-rat IgG (1: 500 dilution) and Rhodamine Red-X-conjugated anti-rabbit IgG (1: 1000 dilution) prepared in 2% GS/PBS.
    - ii. Incubate the coverslips at room temperature (15°C–25°C) for 30 min in a closed container.
  - b. Semi-permeabilized cells.
    - i. Remove the coverslips from the wells of the plates and place them on drops (40  $\mu$ L) of the secondary antibody solution containing DAPI (10,000 times dilution) and Rhodamine Red-X-conjugated anti-rabbit IgG (1000 times dilution) prepared in 2% GS/PBS.

- ii. Incubate the coverslips at room temperature (15°C–25°C) for 30 min in a closed container.
38. Replace the coverslips in the wells with 1 mL of PBS (cell-side up) in the 24-well plates.
39. Wash the coverslips with PBS (1 mL/well) three times.
40. Briefly wash the coverslips with sterilized MilliQ water (1 mL/well) in 24 well plates
41. Mount the coverslips on glass slides with ProLong™ Diamond Antifade Mountant without DAPI.
42. Let the samples dry in a dark place at room temperature (15°C–25°C) overnight (18–20 h).

▮▮ **Pause point:** The slide glass sample can be stored at –20°C until imaging process. Please make sure to defrost before starting observation.

### Imaging and counting the number of GFP-positive LCVs (day 5)

⌚ **Timing:** 2–3 h

We used an inverted microscope (TE2000-U; Nikon) equipped with a digital ORCA-ERA camera (Hamamatsu) to observe the LCVs as described previously (Kitao et al., 2020). Data can be collected using any fluorescent microscope with RGB channels and filters to visualize red, green, and blue signals. A confocal microscope also can be used to observe the LCVs alternatively.

43. Clean coverslips using a paper moistened with water.
44. Set up a glass slide on a 100× oil-immersion objective lens to observe the sample.
45. Observation.
  - a. Cells without semi-permeabilization.
    - i. Find an intracellular *L. pneumophila*, i.e., LCV (positive for red signal, negative for blue signal).
    - ii. Check whether the LCV is associated with green signals (GFP-positive) or not.
  - b. Semi-permeabilized cells.
    - i. Find an LCV (positive for red signal) and DAPI staining (blue signal).
    - ii. Check whether the LCV is associated with green signals (GFP-positive) or not.
46. Count at least 100 LCVs for each sample. Perform statistical analysis using Student's *t* tests for the data obtained from three independent experiments.

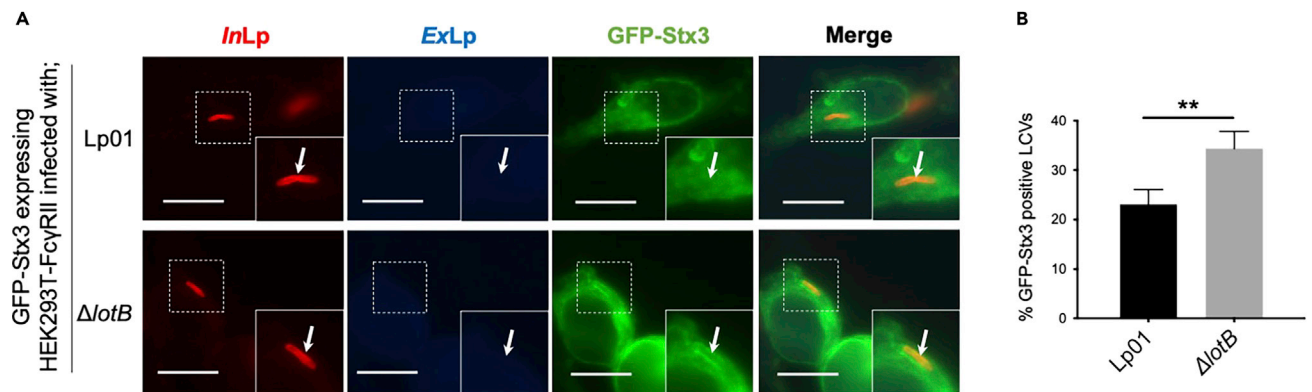
### EXPECTED OUTCOMES

Here, we present results of three experiments performed using this protocol, which include the host t-SNARE GFP-Stx3 and the *L. pneumophila* effector protein LotB. These data are from the original Figure 7 in Kitao et al., 2020.

- Example outcome #1: Result of an analysis to determine whether LotB influences the association of GFP-Stx3 with the LCVs.

Syntaxin 3 (Stx3) is a host t-SNARE protein that is known to form non-canonical SNARE pairing with the v-SNARE Sec22b upon *L. pneumophila* infection (Arasaki and Roy, 2010). Given that the deubiquitinase (DUB) activity of LotB toward Sec22b promotes dissociation of Stx3 from Sec22b during the infection, we examined whether the LotB-mediated dissociation of Stx3 from Sec22b affects the level of Stx3 on the LCVs. As shown in Figure 2, the number of Stx3-positive LCVs in the cells infected with the  $\Delta$ lotB mutant strain was higher than that of the Stx3-positive LCVs in the cells infected with the wild-type strain 4 h after infection.

- Example outcome #2: Result of an analysis using the semi-permeabilization technique to determine whether LotB can induce the release of GFP-Stx3 from the LCVs.



**Figure 2. Example outcome #1: LotB influences the association of GFP-Stx3 with LCVs during *L. pneumophila* infection**

(A) HEK293T-FcγRII cells transiently expressing GFP-Stx3 were infected with the wild-type Lp01 or ΔlotB mutant strains at an MOI of 5. After 4 h, the cells were fixed and visualized using a fluorescence microscope. The number of GFP-Stx3-positive vacuoles containing *L. pneumophila* was assessed. Values represent the means from three independent experiments in which 100 LCVs were assessed. \*\*p < 0.001.

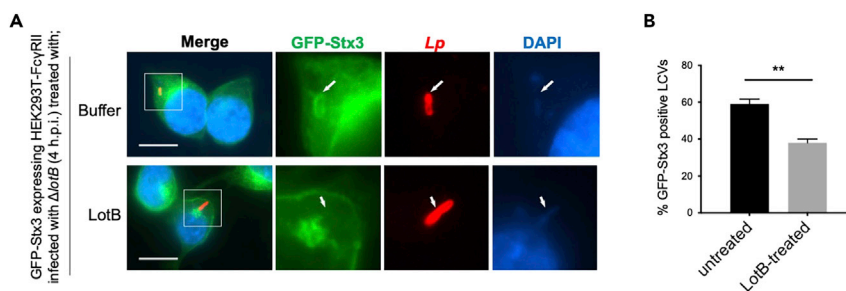
(B) Images of LCVs containing the Lp01 or ΔlotB mutant strains 4 h post infection. Magnified images of the insets are shown at the bottom right of each panel. White arrows indicate LCVs. Scale bars, 10 μm.

We also examined whether Stx3 can physically dissociate from the LCVs due to LotB. We used the purified protein and semi-permeabilized cells after infection (Figure 3). The number of GFP-Stx3-positive LCVs significantly decreased in the cells treated with fulllength LotB compared to the untreated cells, suggesting that LotB induces the release of Stx3 from the LCVs.

- Example outcome #3: Result of an analysis to determine whether LotB can localize to the LCVs.

In this experiment, we assessed whether LotB could localize to the LCV (Figure 4).

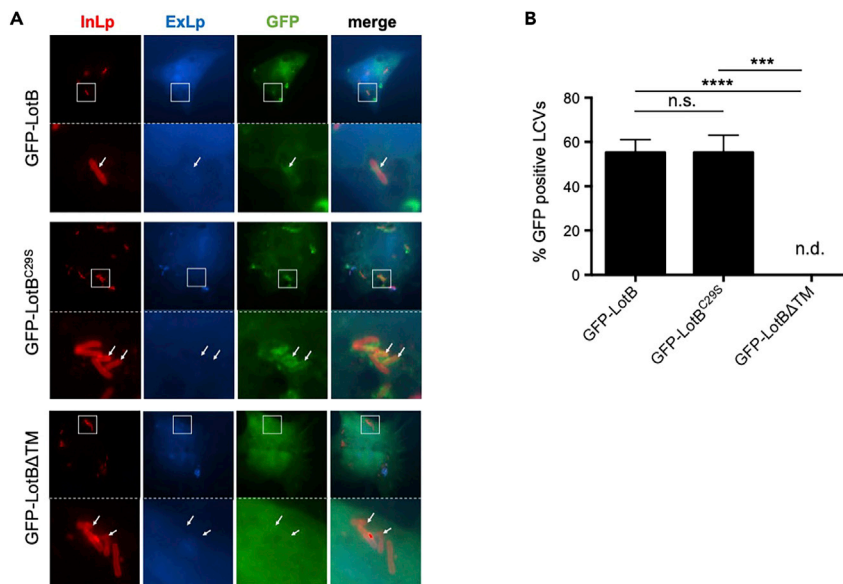
Four hours after infection, ectopically expressed GFP-LotB was readily detected on the LCVs in the presence of nocodazole, which prevents microtubule-dependent vesicular trafficking between the ER-Golgi intermediate compartment (ERGIC) and Golgi, thereby disrupting the later stage of the early secretory pathway. The number of LotB-positive LCVs was not affected by the mutation of catalytic cysteine. However, LotB lacking the transmembrane (TM) domain (GFP-LotBΔTM) was not detected on LCVs, which indicates that the ability of LotB to associate with the LCVs requires the TM domain.



**Figure 3. Example outcome #2 using the semi-permeabilization technique: Influence of LotB on the association of GFP-Stx3 with LCVs during *L. pneumophila* infection**

(A) GFP-Stx3-expressing HEK293T-FcγRII cells were semi-permeabilized with buffer containing digitonin 4 h after infection with the ΔlotB mutant strain at an MOI of 5. After treatment with or without full-length LotB, the cells were fixed and visualized with an immunofluorescence microscope.

(B) Percentages of LCVs positive for the GFP-Stx3 proteins are shown. Values represent means from three independent experiments in which 100 LCVs were assessed. \*\*p < 0.001.



**Figure 4. Example outcome #3: *L. pneumophila* LotB can localize to the LCVs during infection**

(A) HeLa-Fc $\gamma$ RII cells transfected with plasmids expressing GFP-LotB, GFP-LotB<sup>C29S</sup>, or GFP-LotB $\Delta$ TM together with the plasmid expressing FLAG- $\beta'$ -COP were infected with Lp01 at an MOI of 5. After 4 h, the cells were fixed and visualized using a fluorescence microscope. The areas indicated with white squares are magnified and shown in the bottom panels. White arrows indicate the location of LCVs containing *L. pneumophila*. Scale bars, 10  $\mu$ m.

(B) Percentage of GFP-LotB-positive LCVs 4 h after infection. Values represent means from three independent experiments in which 100 LCVs were assessed for each sample. \*\*\*\*p < 0.0001, \*\*\*\*p < 0.00001.

## LIMITATIONS

In this protocol, we have described a method to analyze whether a protein of interest can localize to the LCVs, a vacuole of *L. pneumophila* established during infection in host cells. This protocol can be used to analyze both host proteins and *L. pneumophila* effector proteins. In addition, as an optional experiment, our protocol includes a semi-permeabilization technique to analyze the effect of a target protein on the localization of other proteins of interest. However, these procedures are not specific to *L. pneumophila* and can be used for other intracellular pathogens with proper modifications. For example, selection of a detergent and optimization of the buffer condition to semi-permeabilize the host plasma membrane without disrupting the vacuoles would be a critical factor. For detection of a specific pathogen, antibodies against the target pathogen or alternative detection tools are required.

## TROUBLESHOOTING

### Problem 1

A target protein is not detected on the LCVs (This problem may arise in step 45).

### Potential solution

When analyzing for the first time, whether the target host protein is associated with the LCV, a positive control should be included. GFP-Stx3 can be used as a positive control, but the association of this protein during infection does not depend on the Dot/Icm type IV secretion system (T4SS), which is a bacterial apparatus essential for *L. pneumophila* infection (Arasaki and Roy, 2010). To determine whether your target host protein is recruited to the LCV in a T4SS dependent manner, host v-SNARE Sec22b could act as an alternative positive control protein (Kagan et al., 2004). In addition, you may also consider planning a time-course experiment to see how your target protein behaves at different time points, such as 0.5, 1, 2, 4, and 7 h post infection (step 20).

In the protocol, we used GFP-tagged proteins to monitor cellular localization during infection. Alternatively, other fluorescent protein tags, such as mRFP, can also be used. Protein tagging can alter the function of the protein. In that case, smaller epitope tags, such as FLAG, Myc, or HA, can be used. The combination of an antibody with a target protein and a fluorescence-labeled secondary antibody, such as Alexa Fluor 488, can also be substituted for GFP-tagging of the target protein. Modify the protocol as per the detection system (step 37).

### Problem 2

The fluorescence intensity of GFP-tagged protein is too low or too high (This problem may arise in step 45).

### Potential solution

If the fluorescence intensity of GFP-tagged protein is too low, the amount of plasmid used for transfection needs to be increased (step 15). Also, the lipofection method can be used alternative to PEI. If the fluorescence intensity of GFP-tagged protein is too high, reduction of the amount of plasmid used for transfection may help to improve the signal intensity (step 15).

### Problem 3

An experiment using the *L. pneumophila* isogenic mutant lacking a gene encoding the effector protein of interest revealed that the effector protein influences the subcellular localization of host protein recruited to the LCVs upon infection. However, the semi-permeabilization experiment using the purified effector protein didn't generate the supportive result (This problem may arise in the optional protocols after step 23).

### Potential solution

To influence the subcellular localization of host protein, biochemical events such as the physical interaction between the *Legionella* effector and host protein, the enzymatic activity and the appropriate subcellular localization of the effector protein are considered to be required. If you used partially truncated protein in semi-permeabilization experiment for some reasons, such as to increase the yield of soluble protein, try to use the full-length protein.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tomoe Kitao ([kitao@gifu-u.ac.jp](mailto:kitao@gifu-u.ac.jp)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate and/or analyze any datasets.

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## AUTHOR CONTRIBUTIONS

All authors designed the experiments. T. Kitao optimized and wrote the protocol. T. Kitao prepared the figures. All authors have reviewed the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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