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Running title: Assessment of *Listeria* vacuolar escape

Abstract

Listeria monocytogenes is a bacterial pathogen which invades and multiplies within non-professional phagocytes. Signaling cascades involved in cellular entry have been extensively analyzed, but the events leading to vacuolar escape remain less clear. In this chapter, we detail a microscopy FRET-based assay which allows quantitatively measuring *L. monocytogenes* infection and escape from its internalization vacuole, as well as a correlative light/electron microscopy method to investigate the morphological features of the vacuolar compartments containing *L. monocytogenes*.

Keywords

Listeria monocytogenes, phagocytosis, vacuole, listeriolysin O, phospholipases, CCF4, Förster resonance energy transfer (FRET) microscopy, correlative light/electron microcopy (CLEM)

1. Introduction

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen responsible for listeriosis, a food-borne disease characterized by meningitis in newborns, abortion in pregnant women and septicemia in immunocompromised individuals [1]. For more than 50 years, *Listeria* has been used as major model in infection biology to investigate the interplay between immune cellular responses to control bacterial intracellular pathogens [2] and the role of bacterial virulence factors in subverting host cell functions [3]. *Listeria* internalization within non-phagocytic epithelial cells has been particularly well studied [4, 5]. Interaction of the bacterial surface proteins internalin (InlA) and InlB with their respective host cell receptors E-cadherin and Met triggers the recruitment of a clathrin-based machinery [6, 7] which controls in turn actin recruitment and membrane remodeling at bacterial internalization foci [8]. Modulation of phosphoinositide metabolism cooperates with the actin cytoskeleton to favor [9] or to restrict [10] *Listeria* entry within host cells [11].

Following cell invasion, *Listeria* is located within a membrane-bound compartment that is ruptured to allow bacterial cytoplasmic access. Listeriolysin O (LLO), a pore-forming toxin encoded by the gene *hly* and responsible for the hemolytic activity that was very early associated to virulent *Listeria* strains [12], has been referred as a major actor in disrupting the bacterial vacuole [13, 14]. Two other bacterial virulence factors, PlcA and PlcB, are phosphatidylinositol-specific and broad range phospholipases respectively which also participate in vacuolar destabilization [15, 16]. However, how these bacterial virulence factors specifically contribute to *Listeria* vacuolar escape is not fully understood. For example, inactivation of LLO in J774 cells completely abrogates bacterial translocation to the cytoplasm [14, 17] but in human epithelial HeLa, HEp-2 and Henle 407 cells, the broad range phospholipase PlcB can rescue a Δ*hly* mutant, suggesting that host factors also contribute to the stability of the bacterial vacuole. Indeed, it has been demonstrated in murine macrophages that the gamma-interferon-inducible lysosomal thiol reductase (GILT) and the cystic fibrosis transmembrane conductance regulator (CFTR) enhance the oligomerization and the lytic activity of LLO to facilitate vacuolar rupture [18, 19] while inducible renitence limits vacuolar disruption by restoring the integrity of endolysosomal membranes [20].

Listeria vacuolar escape in epithelial cells has been studied recently by our team [21]. In this present article, we present two complementary approaches to address this topic: we describe first a detailed protocol for a Förster resonance energy transfer (FRET) microscopy assay which measures bacterial cytoplasmic translocation by monitoring the fluorescence of the β-lactamase-sensitive FRET probe CCF4 (this molecule emits photons at 535 nm when intact, but fluoresces at 450 nm when cleaved) (**Figure 1**). The use of an engineered *Listeria* strain expressing a surface β-lactamase allows the identification of vacuolar escape by cleavage of the CCF4 molecule via the surface enzymatic activity. This approach has been successfully used with the bacterial pathogen *Shigella flexneri* to screen multiplexed small interfering (si)RNA libraries for the discovery of cellular factors regulating bacterial cytoplasmic translocation in HeLa cells [22]. We also present a correlative light/electron microscopy (CLEM) method in which we use green fluorescence protein (GFP)-expressing bacteria in conjunction to actin fluorescent staining to assess the localization of internalized bacteria that do not form actin tails: further characterization of these specific bacteria

using transmission electron microscopy allows to investigate in detail their sub-cellular environment, and to analyze their potential association to membrane-bound compartments.

These two methodological strategies are therefore complementary and can be coupled to identify and characterize novel cellular host factors modulating *Listeria* translocation to the cytoplasmic space: the FRET-based microscopy assay is suited to perform high-throughput screens of drug or siRNA libraries targeting host factors, in order to first identify host candidate molecules which may control vacuolar membrane stability (in this manuscript, this assay is coupled to a microscopical test to differentiate extracellular from total bacterial populations, which allows to determine whether failure in vacuolar escape could be explained by inhibition of bacterial entry or vacuolar rupture [21]). The CLEM assay can then be employed to investigate in finer detail the contribution of specific host candidate molecules to the enhancement or inhibition of *Listeria* vacuolar escape, through a morphological analysis of the structure of membranes and intracellular organelles that may interact with the bacterial-containing compartments prior to vacuolar rupture.

2. Materials

Vacuolar escape assays were performed in HeLa cells, which have been commonly used as a model to understand the intracellular lifestyle of *Listeria* [23, 24] and which are dependent on the InlB-invasion pathway. HeLa cells are grown in the absence of antibiotics to avoid potential endocytosis that could kill intracellular bacteria. To increase cellular invasion and therefore the statistical power of the microscopy-based assay described in this chapter, we employ a *L. monocytogenes* EGDe PrfA* strain which contains a Gly145Ser point mutation in the transcriptional regulator PrfA, increasing expression of virulence factors [25]. To express β-lactamase at the bacterial cell surface, we engineered a fusion protein containing the signal peptide of InlA at the N terminal part of the chimera (to direct the protein to the bacterial cell wall), the β–lactamase gene (codon usage optimized for *Listeria*) and the LPXTG motif of InlA at the 3' end of the construct (to anchor the protein to the bacterial cell wall through sortase A). This synthetic gene is under the control of the Hyper-SPO1 constitutive promoter (pHyper) fused to the *hly* 5'-untranslated region to enhance expression of the gene construct [21] and was electroporated into *L. monocytogenes* EGDe PrfA* strain as well as in our negative control *L. innocua* ^{InlB} (synthetic strain containing InlB attached to the cell wall to allow invasion of this nonpathogenic *Listeria* species).

2.1 FRET-based microscopy assay for vacuolar escape quantification.

1. Cell preservation medium: 50% Dulbecco's Modified Eagle Medium (DMEM), 40% heat inactivated fetal calf serum (FCS), 10% DMSO.
2. HeLa cell clone CCL2 (American Type Cell Collection): kept in frozen stocks in cell preservation medium in liquid nitrogen.
3. Standard cell culture medium: DMEM supplemented with 10% heat inactivated FCS.
4. Lipofectamine RNAiMax (ThermoFisher).
5. (Optional) siRNAs (see Section 3).
6. HEPES buffer: 120 mM NaCl, 7 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM Glucose, 25 mM HEPES at pH 7.3.
7. Probenecid (Sigma).
8. CCF4-AM (LiveBlazer Loading Kit, Invitrogen).
9. Solution B: 100 mg/ml Pluronic-F127 surfactant in DMSO/0.1% acetic acid provided along the CCF4-AM LiveBlazer Loading Kit by Invitrogen. Keep at room temperature.
10. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium to rinse chelators from the culture before cell dissociation and used also to prepare dilutions of antibodies
11. Trypsin/EDTA solution.
12. A BHI agar plate with *L. monocytogenes* EGDe PrfA*$^{\beta\text{-lact}}$ colonies.
13. A BHI agar plate with *L. innocua*$^{\beta\text{-lact/InlB}}$ colonies.

14. *Listeria* culture medium: brain-heart infusion (BHI) as liquid medium or in agar plates. Chloramphenicol was used at a final concentration of 7 µg/ml to maintain the pPl2 plasmid encoding the β-lactamase and Erythromycin 5 µg/ml was used to maintain the pP1 plasmid encoding the InlB in *L. innocua*.
15. Dark 96-well cell culture plates for cell microscopy.
16. Distilled water.
17. Fixation solution: DPBS, 4% paraformaldehyde (PFA) 1mM probenecid.
18. DPBS supplemented with 1% bovine serum albumin (BSA).
19. DPBS supplemented with 1% BSA and 0.1%Triton X-100.
20. Anti-*L. monocytogenes* and anti-*L. innocua* polyclonal rabbit antibodies (*see* **Note 1**).
21. Fluorescently labeled secondary antibodies. Goat anti-rabbit Alexa 647 and Goat anti-rabbit Alexa 488 (LifeTechnologies).
22. Dye Draq5 (Ebioscience) and Hoechst (Thermo-Fisher) to stain cell nuclei and DNA (bacteria and host).
23. Automated confocal microscope Opera QEHS (PerkinElmer Technologies) (*see* **Note 2**).
24. Multichannel pipette.
25. 50 ml reagent reservoir.

2.2 CLEM assay for ultrastructural analysis.

1. MatTek petri dishes with gridded coverslip (P35G-2-14-C-GRID).
2. Standard cell culture medium: DMEM, 10% heat-inactivated fetal calf serum.
3. Infection cell culture medium: DMEM, 1% heat-inactivated fetal calf serum.
4. HeLa cell clone CCL2 (American Type Cell Collection): kept in frozen stocks in cell preservation medium in liquid nitrogen.
5. Brain-heart infusion agar plate.
6. *L. monocytogenes* strain EGDe PrfA*-green fluorescent protein (GFP).
7. Gentamicin solution.
8. Fixation solution 1: PHEM buffer, 4% PFA.
9. Anti-*L. monocytogenes* primary antibodies (*see* **Note 1**).
10. DAPI or Hoechst, 1mg/ml.
11. Fluorescently labeled secondary antibodies (anti-rabbit AlexaFluor 647 antibodies).
12. Fluorescently labeled phalloidin (AlexaFluor 546).
13. Phosphate-buffered solution (PBS) supplemented with 1% BSA.
14. Microscope equipped with fluorescence/white light lamps and 20x/100x objectives.
15. Fixation solution 2: PHEM buffer, 2.5% glutaraldehyde.
16. Pen and nail polish.
17. 0.1 M cacodylate buffer.
18. 1% osmium-tetroxid solution in H_20.
19. 70% ethanol supplemented with 1.2% uranylacetate.
20. Distilled H_20.

21. Series of graded ethanol: 50%, 75%, 95% and 100% ethanol.
22. EPON A and EPON B resin.
23. Gelatine capsules.
24. Razor blades.
25. Scalpel.
26. Glass slides.
27. Incubator at 60°C.
28. Matches.
29. Reynold's lead citrate.
30. 80-120 kV transmission electron microscope equipped with a CCD camera.
31. Diamond knife.
32. Carbon-coated grids.
33. 4% uranyl acetate in H20.

3. Methods

The FRET-based microscopy assay for vacuolar escape is divided in two parts: in the first part (3.1.1) cells are pre-loaded with the CCF4 dye and are subsequently infected with *Listeria* for 1 hour. Afterwards, nuclei are stained with the Draq5 probe and the cells are imaged in an automated confocal microscope to identify the ratio of the 450 nm versus the 535 nm signals, which allows estimating the number of cells in which bacteria are trapped in vacuoles versus those that translocate to the cytoplasm. In the second part (3.1.2) of the assay, extracellular and total *Listeria* are differentially labeled to estimate the number of bacteria which reached the intracellular space. The CLEM protocol is also divided in two parts: in the first part (3.2.1) mammalian cells are infected with *Listeria* and labeled to detect extracellular bacteria, nuclei, the actin cytoskeleton and events of interest (intracellular bacteria not associated with actin) and finally imaged on a fluorescence microscope. In the second part (3.2.2) of the assay, cells are embedded in a resin, the events of interest are located, the resin is trimmed and contrasted, and finally the cells are viewed on a transmission electron microscope.

As mentioned in the introduction, this FRET-based assay is suited to screen drug or siRNA libraries and in the protocol we present below, we describe a methodology which includes cellular transfection with siRNAs in a 96-well plate format. However, this protocol can be used without siRNAs and can be downscaled to a 24-well plate format if required. In the same manner, the CLEM assay that we present here in the absence of siRNA transfection, can be coupled to siRNA treatment to monitor the specific phenotype of targets identified in the FRET-based assay.

3.1 FRET-based microscopy assay for vacuolar escape quantification.

3.1.1. CCF4 loading, *Listeria* infection and FRET measurements.

1. Perform the siRNA transfection of HeLa cells 72h before the infection for efficient knock down of gene expression (*see* **Note 3**). For each well mix 1 μl of the siRNA under study (diluted at 2μM) with 9.0 μl of DMEM without serum and mix gently (*see* **Note 4**). Mix gently 0.1 μl of lipofectamine RNAiMax with 9.9 μl of DMEM and wait for 5 minutes. Combine the diluted siRNA with the diluted lipofectamine, mix gently and incubate 30 min at room temperature. Add these 20 μl of siRNA/lipofectamine to one well of the 96-well plate black microscopy plate (**Figure 2**) and plate 6000 HeLa cells in a volume of 80 μl (*see* **Note 5**), avoiding the use of wells in the borders of the plate (columns 1 and 12, rows A and H) (*see* **Note 6**). After distribution of the cells with multichannel pipette, allow the cells to settle down for 15 min at room temperature (*see* **Note 7**).

2. Incubate the plate at 37°C in a humidified 10% CO_2-containing atmosphere incubator (*see* **Note 8**).

3. The day before the infection prepare a liquid culture of *L. monocytogenes* EGDe PrfA*$^{\beta\text{-lact}}$ (include chloramphenicol) and *L. innocua*$^{\beta\text{-lact/InlB}}$ (include chloramphenicol and erythromycin) by inoculating a bacterial colony from the agar plate to 5mL of BHI liquid medium supplemented with the required antibiotics. Allow the culture to grow during 18h at 37°C in a shaker (180 rpm).

4. The day of infection remove the cell culture medium and wash the cells with 100 µl DPBS per well. Remove the DPBS. (*see* **Note 9**).

5. Load the cells with CCF4-AM. Prepare the CCF4-AM loading mix for a final volume of 25 µl per well containing 1 mM probenecid, 1.0 µM CCF4-AM and 1.25 µl of loading solution B in EM buffer (*see* **Note 10**). Allow cells to load with CCF4-AM for 150 minutes at room temperature in the dark.

6. Prepare bacteria for infection. Spin 0.5 mL of the overnight *Listeria* cultures at 5,500 x *g* for 3 min using a tabletop centrifuge and remove the supernatant (containing LLO which is cytolytic). Resuspend the pellet in 1ml of DPBS. Repeat the washing steps to a total of four washes. After the last wash, resuspend bacteria in 1mL of DPBS.

7. Estimate the number of bacteria by reading the bacterial optical density at 600 nm (for *Listeria* spp., OD=1 is equivalent of 1 x 10^9 bacteria/ml). Prepare the inoculum by adding the necessary volume of the bacteria suspension to a final volume of 100 µl of EM buffer/1 mM probenecid per well (*see* **Note 11**).

8. After 150 min of incubation discard the CCF4-AM loading solution from the 96-well plate and wash the HeLa cells once with 150 µl PBS/1 mM probenecid (*see* **Note 12**).

9. Discard the PBS/1 mM probenecid and add 100 µl of the inoculum in EM buffer/1 mM probenecid to each well.

10. Centrifuge the 96-well plate at 210 x *g* during 3min at room temperature to synchronize the infection.

11. Switch the plate to an incubator with a humidified 10% CO2 atmosphere at 37 °C for 1h to allow *L. monocytogenes* EGDe PrfA*$^{\beta\text{-lact}}$ and *L. innocua*$^{\beta\text{-lact/InlB}}$ invade cells.

12. Remove the plate from the incubator and wash cells with 150 µl PBS/1 mM probenecid.

13. Discard the DPBS/1mM probenecid and fix with 50 µl PFA 4%/1 mM probenecid for 10 min in the dark (*see* **Note 13**).

14. Eliminate the PFA 4%/1 mM probenecid and wash the plate with 150 µl DPBS.

15. Remove the DPBS and add 30 µl of 10 µM nuclei dye Draq5. Incubate during 30 min in the dark.

16. Discard the Draq5 solution and wash with 150 µl DPBS.

17. Eliminate the DPBS and leave samples in 100 µl of new DPBS.

18. Acquisition of fixed samples is performed using the automated spinning disk confocal microscope Opera QEHS (PerkinElmer Technologies). The following sequence is used: firstly, CCF4 (excitation/emission [ex/em] 405/535 and 405/450 nm on two separate cameras) and secondly, Draq5 (ex/em 640/690). 23 fields, correspond roughly to 6000 cells, are acquired per well using a 10x air objective (numerical aperture: 0.4). Recorded images (**Figure 3**) are transferred to a Columbus database (PerkinElmer Technologies) for storage, management and analysis (*see* **Note 14**).

19. Feature extraction was performed for each individual cell by using integrated Columbus building block routines (*see* **Note 14**). In brief, cells were segmented using the Draq5 channel by first detecting nuclei and then expanding into the cytoplasm for robust single cell identification.

20. The FRET 450/535 ratio is calculated for each cell and a mean ratio is calculated per well.

21. Statistical analysis of the results is performed using the strictly standardized mean difference (SSMD) statistical tests for quality control (QC), hit selection and validation [22].

22. If the experiment is not continued immediately (see Section 3.1.2), keep the plate at 4°C for not more than 24 h (*see* **Note 15**).

3.1.2. Differential staining of extracellular and total *Listeria* populations.

1. Continuing with samples from Section 3.1.1, discard the DPBS contained in the wells of the plate.

2. Add 100 µl of new DPBS and discard it again.

3. Extracellular *Listeria* spp. are labeled with a primary polyclonal rabbit anti-*L. monocytogenes* or anti *L. innocua* serum (*see* **Note 1**). Prepare this primary antibody solution by diluting the antibodies 1:500 in DPBS supplemented with 1% serum albumin. Add 30 µl of primary antibody to each well.

4. Incubate for 30 min at room temperature to label extracellular *Listeria*.

5. Discard the primary antibody solution and wash four times with 100 µl of DPBS supplemented with 1% serum albumin.

6. Dilute the secondary goat anti-rabbit Alexa 647 1:200 in DPBS supplemented with 1% serum albumin, and add 30 µl to each well.

7. Incubate for 30 min at room temperature in the dark to avoid degradation of the fluorescent probe.

8. Discard the secondary antibody solution and wash four times with 100 µl of DPBS supplemented with 1% serum albumin.

9. Cells are permeabilized using 100 µl of 0.1%Triton X-100 for 4 min at room temperature in the dark.

10. Discard the Triton X-100 solution and wash one time with 100 µl of DPBS supplemented with 1% serum albumin.

11. Intracellular (and extracellular) *Listeria* spp. are labeled with a primary polyclonal rabbit anti-*L. monocytogenes* or anti *L. innocua* serum. Prepare this primary antibody solution by diluting the antibodies 1:500 in DPBS supplemented with 1% serum albumin. Add 30 µl of primary antibody to each well.

12. Incubate for 30 min at room temperature in the dark to label total *Listeria* populations.

13. Discard the primary antibody solution and wash four times with 100 µl of DPBS supplemented with 1% serum albumin.

14. Dilute the secondary goat anti-rabbit Alexa 488 1:200 in DPBS supplemented with 1% serum albumin containing Hoechst diluted 1:1000. Add 30 µl to each well.

15. Incubate for 30 min at room temperature in the dark to avoid degradation of the fluorescent dyes.

16. Discard the secondary antibody solution and wash four times with 100 µl of DPBS. Keep the cells in 100 µl of DPBS at 4°C in the dark until acquisition.

17. Acquisition of fixed samples is performed four days after the immunofluorescence (*see* **Note 16**) using the automated spinning disk confocal microscope Opera QEHS (PerkinElmer Technologies) (*see* **Note 2**). The following sequence is used: extracellular

bacteria labeled with Alexa 647 and Alexa 488 secondary antibodies (ex/em 640/690), and intracellular bacteria labeled only with an Alexa 488 secondary antibody (ex/em 488/540) and Hoechst 33342 (ex/em 405/450). 23 fields, corresponding roughly to 6000 cells, are acquired per well using a 10x air objective (numerical aperture: 0.4). Recorded images (**Figure 4**) are transferred to a database for storage, management and analysis.

18. The number of intracellular bacteria per cell was measured using Columbus analyzing building block routines (*see* **Note 14**).

19. Statistical analysis of the results is performed using the strictly standardized mean difference (SSMD) statistical tests for quality control (QC), hit selection and validation [22].

20. Classify each hit by cross-examination of the two screening results, i.e. a hit selected in the two assays defines a “bacteria entry hit”, a hit in the FRET assay but not in the “intra/extra bacteria” assay corresponds to a “vacuolar rupture hit”.

3.2 CLEM assay of intracellular *Listeria* populations.

3.2.1. *Listeria* infection and immunofluorescence.

1. HeLa cells and *L. monocytogenes* EGDe.PrfA*-GFP are grown as described in steps 3 to 7 of Subheading 3.1.1 of the FRET-based vacuolar escape analysis protocol (bacterial agar plates and liquid medium should be supplemented with 5 μg/ml of erythromycin to select for GFP-expressing clones).

2. Prepare HeLa cells for the experiment using similar procedures as the ones described in steps 1 to 3 of Subheading 3.1.1, plating cells in MatTek petri dishes (instead of dark microscopy 96-well plates) (**Figure 5)** (*see* **Note 17**).

3. Infect cells as described in Subheading 3.1.1, steps 6-10, using infection medium (DMEM 1% FCS).

4. Transfer the MatTek petri dishes to an incubator with a humidified 10% $C0_2$ atmosphere at 37°C, and let the bacteria enter the cells for 1 h.

5. Aspirate the infection medium, wash cells twice with 2 ml of pre-warmed cell culture medium supplemented with 40 μg/ml gentamicin and replace with 2 ml of the same gentamicin-containing medium.

6. Transfer the MatTek petri dishes back to the incubator, and keep them at 37°C in the humidified 10% $C0_2$ atmosphere for another 4 hours (*see* **Note 18**).

7. Aspirate the gentamicin-containing medium, wash cells twice with 2ml of pre-warmed standard cell culture medium.

8. Adjust medium level to 1ml, and fix cells by adding 1ml of the 4% PFA/PHEM buffer fixation solution (final PFA concentration: 2%) for 10 min (*see* **Note 19**).

9. Replace the fixation solution by 1ml of 4% PFA/PHEM buffer fixation solution and fix for another 20 min at room temperature.

10. Remove the fixative, wash the cells three times with 2 ml of PBS supplemented with 1% bovine serum albumin.

11. Prepare the primary antibody solution by diluting the rabbit-derived anti-*L. monocytogenes* antibody (*see* **Note 1**) 1:500 in DPBS supplemented with 1% bovine serum albumin, and add 500 *µ*l of primary antibody per dish (only in the center area).

12. Incubate for 20 min at room temperature to label extracellular *L. monocytogenes*.

13. Discard the primary antibody solution and wash 4 times with 2 ml of DPBS supplemented with 1% bovine serum albumin.

14. Dilute the secondary AlexaFluor 647-coupled anti-rabbit antibody (1:200), the DAPI solution (1:1000) and the AlexaFluor 546-coupled phalloidin in PBS supplemented with 1% bovine serum albumin.

15. Add 500 *µ*l of this secondary probe solution to each dish (use only the center area) (*see* **Note 20**).

16. Incubate for 30 min at room temperature in the dark to protect the fluorescent antibody.

17. Discard the secondary probe solution and wash 4 times with 2 ml of DPBS supplemented with 1% bovine serum albumin.

18. Perform imaging in an inverted microscopy using fluorescence and a 100x objective to identify cells in which GFP-positive bacteria are not detected by the secondary AlexaFluor 647-coupled antibody and in which actin comet tails are not detected by the AlexaFluor 546-coupled phalloidin.

19. Locate the position of the cells of interest in the grid by imaging with white light and a 20x objective (*see* **Note 21**).

3.2.2. Sample processing for electron transmission microscopy.

20. Fix the cells a second time by adding 1 ml of the PHEM buffer/glutaraldehyde fixation solution for 20 min.

21. Wash the samples three times for 5 min with PHEM buffer to remove the glutaraldehyde.

22. When the glutaraldehyde is removed, mark the areas with the cells of interest with a pen and nail polish (*see* **Note 22**).

23. Post-fix the samples for 45 min with 1% OsO_4 in the dark.

24. Wash the samples three times for 5 min with H_20.

25. Incubate the samples 5 min with the 25% ethanol solution.

26. Incubate the samples 5 min with the 50% ethanol solution.

27. Incubate the samples 20 min with the 70% ethanol solution supplemented with 1.2% uranyl acetate.

26. Prepare the EPON solution according to the manufacturer's instructions (*see* **Note 23**).

29. Incubate the samples 5 min with the 75% ethanol solution.

30. Incubate the samples two times for 10 min each time with the 95% ethanol solution.

31. Finally incubate the samples three times for 6 min each time with the 100% ethanol solution.

32. Put a drop of the mixed EPON solution on a glass slide.

33. Remove carefully the coverslip from the petri dish with a scalpel and put it on top of the EPON drop with the cells facing up.

34. Put a drop of EPON on the cells and incubate for at least 6 h at room temperature.

35. Fill a capsule of gelatin with EPON and place it in such a way that the mark with the cells of interest is located at the center (**Figure 6**).

36. Incubate overnight at room temperature and then polymerize the samples for 48 hours at 60°C.

37. After the polymerization, the EPON is removed from the coverslip by heating the glass slide with a match.

38. The area of interest is trimmed with a trimming diamond or a razor blade (*see* **Note 24**).

39. Thin sections (70 mm nominal thickness) are prepared and picked up with slot grids.

40. Stain the sections with 4% uranyl acetate and Reynold's lead citrate.

41. Observe the samples in a transmission electron microscope operated between 80-120kV (*see* **Note 25**).

4. Notes

1. In our experiments, we use homemade rabbit polyclonal antibodies against *L. monocytogenes* and *L. innocua*. There are many commercial monoclonal and polyclonal antibodies that can be used to specifically recognize *L. monocytogenes*, and there are several commercial polyclonal antibodies that recognize several *Listeria* spp. and therefore can be used to recognize both *L. monocytogenes* and *L. innocua.* When using these primary antibodies, special attention should be taken concerning the species in which they are produced, in order to choose the right secondary antibodies.

2. We present in this document results generated using an automated confocal microscopy. However, a non-automated wide-field microscopy equipped with a motorized stage can be also used. The only special requirement is the right combination of filters to detect FRET: excitation is performed at 405 nm (Semrock, FF01-387/11-25) and emission detected via 450 nm (Semrock, FF02-447/60-25) and 535 nm (Semrock, FF01-520/35-25) filters.

3. If the aim of the experiment does not require transfection of siRNAs (for example, for the analysis of the specific contribution of bacterial proteins in vacuolar escape), HeLa cells can be seeded into the 96-well plate 15-24 h before infection [26].

4. Positive controls of siRNA transfection and gene knock-down are strongly suggested. We suggest the use of siRNAs targeting the Kinesin Family Member 11 (Kif11) that arrest cells in mitosis, leading to reduced cell numbers exhibiting a ‘rounded-up’ phenotype.

5. We typically use quadruplicates for each siRNA in order to obtain statistically significant results, but the number of well replicates per siRNA is flexible. We recommend preparing four consecutive wells in the same column for each siRNA. It is important to check the number of cells per well the day of infection in order to test if the siRNAs under study cause cell death.

6. The wells in the borders of the plate (columns 1 and 12, rows A and H) are not used in order to avoid interferences due to evaporation. We therefore fill these wells with culture medium that will serve as evaporation and temperature buffer for inner wells.

7. The position of the wells in a plate can cause artifacts. Some possibilities to avoid these artifacts include: 1) allow cells to settle down for 15 min at room temperature 2) Maintain the plates in the incubator on aluminum blocks to ensure equal distribution of the temperature across the plate 3) Seal the plates with parafilm to prevent evaporation and 4) Distribute randomly the control wells as well the rest of the siRNA under study.

8. Take always the plate by the lateral edges. Do not touch the bottom of the plate with your fingers to avoid smearing which may affect image acquisition.

9. Remove the liquid from the wells by inverting the plate gently but efficiently. This technique avoids detachment of cells from the well. Always settle the different reagents in the well very carefully by letting the liquid fall slowly through the wall of the wells, otherwise cells will detach from the bottom.

10. Pipet slowly to avoid bubble formation and inconsistency in the distribution of the CCF4-AM loading solution.

11. We normally use a *L. monocytogenes* PrfA* strain and a multiplicity of infection (MOI) of 10. Using a higher MOI (i.e 20) does increase the statistical power of the assay but could cause undesirable cytotoxic effects by virulence factors secreted by this bacterium. It could be recommendable to increase the MOI if a non-PrfA* strain is used.

12. The continuous use of probenecid is critical to avoid leakage of the CCF4 out of the cells.

13. PFA is toxic and must be handled in an appropriate fume hood. After cell fixation PFA should be disposed of correctly in a sealed container.

14. For this manuscript, we used the Columbus database (PerkinElmer Technologies) for image storage, management and analysis. However, any storage device or database including the Open Microscopy Environment repository OMERO (http://www.openmicroscopy.org/site/products/omero) can be used instead. For image analysis, open resources including ImageJ ((http://imagej.nih.gov/ij/), FIJI (http://fiji.sc/Fiji), Icy (http://icy.bioimageanalysis.org/) and CellProfiler (http://cellprofiler.org/) are also excellent solutions.

15. PFA-fixed cells get permeabilized after 24h. For this reason, the differential immuno-staining of total versus extracellular bacteria should be performed before 24h post PFA fixation.

16. To avoid an interference signal from the CCF4 probe, the cells are kept at 4°C during four days to allow the passive leakage of the probe from the cell cytoplasm.

17. MatTek petri dishes contain a grid on the coverslip as indicated in **Figure 5**. The characters and numbers illustrated in the grid are photo-etched into the coverslip and therefore leave an imprint on the EPON resin that will be used to embed the cells, allowing locating them during the resin trimming. If the coverslip is mounted upside-down, no imprint will be left on the EPON and it will not be possible to locate the cells. Therefore, verify that the MatTek petri dishes have a grid in the right orientation: the characters and letters are not inverted when looking at the dish on a standard white light microscope.

18. In this assay, GFP bacteria of interest will be located by the absence of extracellular staining, and by the absence of actin staining. We would need therefore to infect cells long enough (at least

4 hours) to allow for actin polymerization in those bacteria that escape their vacuolar containing compartment.

19. Mixing the fixation solution with the medium containing the cells is a gentler way of fixing them, allowing to better preserve their morphological features.

20. Use only the central area of the dish to avoid the use of large volumes of antibodies or secondary probes).

21. To locate at least two independent events within a MatTek dish, it is recommended to choose cells which are not close to each other and which are located preferentially in separate areas of the grid (for example, one event may be located in the area with numbers and characters while the other event may be located in the area which contain only characters).

22. The cell of interest can be localized on a basic, inverted light microscope that has phase contrast. A 4x objective gives sufficient space to mark with a pen the area on the grid from underneath the coverslip. Since most markers dissolve in epon resin, we recommend to put on the mark a fine drop of nail polish.

23. The hardness of the Epon mixture depends on the ratio of the hardeners in the mixture. We recommend to use a hard formula, which facilitates easier detachment of the coverslip.

24. It is important to have the area to be sectioned as small as possible. In addition, the orientation of the sample/cell of interest in the trimmed area should be noted: this helps to get orientated when the sections are observed.

25. Since the protocol does not include fiducial markers, the correlation between the light microscopy image of a whole cell and the EM image of a thin section needs to be done manually by eye. It is important to know the orientation of the cell of interest and the neighbors within the trimmed trapeze. We recommend to start the observation not with the first grid, which contains the sections of the cells close to the coverslip, but rather with a grid that that contains sections where they cells are cut in the medium of their nucleus. At low/intermediate magnification the nuclei are clearly recognizable as large organelles (**Figure 7**). Their pattern can be correlated to the Hoechst/Dapi staining in immunofluorescence. Once the cell of interest is found in the section, the other sections/grids are screened to find the bacteria/events of interest. Dehydration at room temperature is fast but induces artifacts to membranes. In case the membrane of the bacteria containing vacuole is not clearly visible or not perpendicular to the surface of the section, we use the presence of ribosomes or other cytoplasmic material as criteria to judge the state of vacuole (**Figure 8**).

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5. References

1. de Noordhout C M, Devleesschauwer B, Angulo F, et al. (2014) The global burden of listeriosis: a systematic review andmeta-analysis. The Lancet Infectious Diseases 14:1073–1082.

2. Mackaness GB (1962) Cellular resistance to infection. J Exp Med 116:381–406.

3. Cossart P (2011) Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. Proceedings of the National Academy of Sciences. doi: 10.1073/pnas.1112371108

4. Pizarro-Cerdá J, Kühbacher A, Cossart P (2012) Entry of Listeria monocytogenes in mammalian epithelial cells: an updated view. Cold Spring Harb Perspect Med. doi: 10.1101/cshperspect.a010009

5. Kühbacher A, Emmenlauer M, Rämö P, et al. (2015) Genome-Wide siRNA Screen Identifies Complementary Signaling Pathways Involved in ListeriaInfection and Reveals Different Actin Nucleation Mechanisms during ListeriaCell Invasion and Actin Comet Tail Formation. mBio 6:e00598–15. doi: 10.1128/mBio.00598-15

6. Bonazzi M, Kühbacher A, Toledo-Arana A, et al. (2012) A Common Clathrin-Mediated Machinery Co-ordinates Cell-Cell Adhesion and Bacterial Internalization. Traffic 13:1653–1666. doi: 10.1111/tra.12009

7. Pizarro-Cerdá J, Bonazzi M, Cossart P (2010) Clathrin-mediated endocytosis: What works for small, also works for big. Bioessays 32:496–504. doi: 10.1002/bies.200900172

8. Pizarro-Cerdá J, Cossart P (2009) Listeria monocytogenes membrane trafficking and lifestyle: the exception or the rule? Annu Rev Cell Dev Biol 25:649–670. doi: 10.1146/annurev.cellbio.042308.113331

9. Ireton K, Payrastre B, Chap H, et al. (1996) A role for phosphoinositide 3-kinase in bacterial invasion. Science 274:780–782.

10. Kühbacher A, Dambournet D, Echard A, et al. (2012) Phosphatidylinositol 5-phosphatase oculocerebrorenal syndrome of Lowe protein (OCRL) controls actin dynamics during early steps of Listeria monocytogenes infection. Journal of Biological Chemistry 287:13128–13136. doi: 10.1074/jbc.M111.315788

11. Pizarro-Cerdá J, Kühbacher A, Cossart P (2014) Phosphoinositides and host-pathogen interactions. Biochim Biophys Acta. doi: 10.1016/j.bbalip.2014.09.011

12. Seastone CV (1935) Pathogenic organisms of the genus *Listerella.* J Exp Med 62:203–212.

13. Gaillard JL, Berche P, Mounier J, et al. (1987) In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. Infect Immun 55:2822–2829.

14. Henry R, Shaughnessy L, Loessner MJ, et al. (2006) Cytolysin-dependent delay of vacuole maturation in macrophages infected with Listeria monocytogenes. Cell Microbiol 8:107–119. doi: 10.1111/j.1462-5822.2005.00604.x

15. Mengaud J, Braun-Breton C, Cossart P (1991) Identification of phosphatidylinositol-specific phospholipase C activity in Listeria monocytogenes: a novel type of virulence factor? Mol Microbiol 5:367–372.

16. Vázquez-Boland JA, Kocks C, Dramsi S, et al. (1992) Nucleotide sequence of the lecithinase operon of Listeria monocytogenes and possible role of lecithinase in cell-to-cell spread. Infect Immun 60:219–230.

17. Barry RA, Bouwer HG, Portnoy DA, Hinrichs DJ (1992) Pathogenicity and immunogenicity of Listeria monocytogenes small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect Immun 60:1625–1632.

18. Singh R, Jamieson A, Cresswell P (2008) GILT is a critical host factor for Listeria monocytogenes infection. Nature 455:1244–1247. doi: 10.1038/nature07344

19. Radtke AL, Anderson KL, Davis MJ, et al. (2011) Listeria monocytogenes exploits cystic fibrosis transmembrane conductance regulator (CFTR) to escape the phagosome. Proceedings of the National Academy of Sciences 108:1633–1638. doi: 10.1073/pnas.1013262108

20. Davis MJ, Gregorka B, Gestwicki JE, Swanson JA (2012) Inducible Renitence Limits Listeria monocytogenes Escape from Vacuoles in Macrophages. J Immunol 189:4488–4495. doi: 10.4049/jimmunol.1103158

21. Quereda JJ, Pizarro-Cerdá J, Balestrino D, et al. (2015) A Dual Microscopy-Based Assay to Assess Listeria monocytogenesCellular Entry and Vacuolar Escape. Appl Environ Microbiol AEM.02302–15. doi: 10.1128/AEM.02302-15

22. Mellouk N, Weiner A, Aulner N, et al. (2014) Shigella Subverts the Host Recycling Compartment to Rupture Its Vacuole. Cell Host Microbe 16:517–530. doi: 10.1016/j.chom.2014.09.005

23. Mostowy S, Janel S, Forestier C, et al. (2011) A Role for Septins in the Interaction between the Listeria monocytogenes Invasion Protein InlB and the Met Receptor. Biophys J 100:1949–1959. doi: 10.1016/j.bpj.2011.02.040

24. Quereda JJ, Pucciarelli MG (2014) Deletion of the membrane protein Lmo0412 increases the virulence ofListeria monocytogenes. Microbes and Infection 16:623–632. doi: 10.1016/j.micinf.2014.07.002

25. Ripio MT, Domínguez-Bernal G, Lara M, et al. (1997) A Gly145Ser substitution in the transcriptional activator PrfA causes constitutive overexpression of virulence factors in Listeria monocytogenes. J Bacteriol 179:1533–1540.

26. Kühbacher A, Cossart P, Pizarro-Cerdá J (2014) Internalization Assays for Listeria monocytogenes. In: Methods in Molecular Biology. Springer New York, New York, NY, pp 167–178.

Figure Legends

Figure 1. FRET-based assay principle for tracking vacuolar rupture. CCF4-AM diffuses through the plasma membrane into the cytoplasm where it is modified by cell esterases to form CCF4. CCF4 is trapped in the cytoplasm emitting photons at 535 nm when intact, but fluorescing at 450 nm when cleaved. *L. monocytogenes* encoding a surface β-lactamase enters into cells, escape from its vacuolar compartments and cleaves the CCF4 probe whilst *L. innocua*^{inlB} encoding a surface β-lactamase is able to enter into cells but remain trapped inside vacuoles.

Figure 2. 96-well dark microscopy plate. A. Frontal and **B.** side view of plates used for the FRET-based microscopy assay, which should be dark to preserve the fluorescence signals throughout the experiment. In this particular case, a Costar plate is depicted.

Figure 3. Assessment of *L. monocytogenes* vacuolar escape using a FRET-based microscopical assay. HeLa cells were loaded with CCF4-AM and infected with *L. monocytogenes* EGDe PrfA*$^{\beta\text{-lact}}$ (**A**) or *L. innocua*$^{\beta\text{-lact/InlB}}$ (**B**) strains for 1 hr. Images were obtained using a confocal microscope OPERA QEHS with a 10x objective. Pictures were obtained after merging the following channels: 535 nm (green, intact CCF4 probe) and 450 nm (blue, cleaved CCF4) (Bar: 100 μm).

Figure 4. Immunofluorescence staining to differentiate extracellular versus total *L. monocytogenes*. HeLa cells previously loaded with CCF4-AM and assessed for vacuolar escape were subjected to immunofluorescence. Extracellular bacteria were marked with a secondary goat anti-rabbit Alexa 647 (red) and total (extracellular and intracellular) bacteria were marked with a secondary goat anti-rabbit Alexa 488 (green) after cell permeabilization with 0.1% triton X-100. Nuclei were stained with Hoechst (blue). Intracellular and extracellular bacteria are labeled respectively in green and red (Bar: 100 μm).

Figure 5. MatTek 35 mm dish. A. Frontal and side views of a MatTek 35 mm dish, with a glass coverslip that includes a photo-etched grid. **B.** Scheme of the grid photo-etched on the glass coverslip.

Figure 6. Sample preparation for transmission electron microscopy. A. The gridded coverslip with the cells/events of interest is removed from the dish and flat-embedded in Epon. **B.** After polymerization, Epon is removed from the coverslip by heating the glass slide with a match. **C.** Close-up of the flat-embedded sample after coverslip removal. **D.** The Epon is cut to separate the two gelatine capsuls and one is mounted on the sample holder of the microtome. **E.** Close-up of the region of interest before trimming. **F.** Rough trimming of the area of interest before trimming the trapezoid. **G.** Trapezoid-shape sample after trimming. Note that only the area above the top of the 1 remains from the square 1N. **H.** Cut thin sections floating on the water in the boat of the knife. **I.** Thin sections on a slot grid. **J.** Low magnification of the thin sections on the slot grid after contrasting.

Figure 7. CLEM of HeLa cells infected with *L. monocytogenes* GFP. A. Low magnification (40x) of a monolayer of HeLa cells growing on a MatTek dish. The cell of interest (dashed box) is located at the left lower corner of the eN square (tilted -80°C in the image) (Bar: 10 *μ*m). **B.** DAPI staining of the same monolayer of HeLa cells depicted in **A.** (N: nuclei). **C.** Actin staining at high magnification (63x) of the cell of interest located in the dashed box in **A.** and **B.** The dashed boxes labelled 1, 2 and 3 represent three individual groups of bacteria that will be observed in detail by electron microscopy (**Figure 8**). Actin polymerizing bacteria can be clearly observed in groups 1 and 3. (Bar: 10 *μ*m). **D.** Same area as in **C.** displaying the *L. monocytogenes* GFP signal (Bar: 10 *μ*m).. **E.** Same area as in **C.** displaying the antibody labelling against extracellular *L. monocytogenes* (Bar: 10 *μ*m). **F.** DAPI staining of the same area as in **C. G.** Merge of images from **C.** to **F.**: actin is displayed in red, *L. monocytogenes* GFP are shown in green, extracellular *L. monocytogenes* are displayed in blue and DAPI staining is shown in white (Bar: 10 *μ*m). **H.** Transmission electron micrograph of the cell observed in **G.** (Bar: 10 *μ*m). In the dashed box 2, the bacterium pointed with a white arrow-head (**G.**) and a black arrow head (**H**.) is present in a vacuole (**Figure 8**).

Figure 8. Transmission electron microscopy of HeLa cells infected with *L. monocytogenes* GFP. 1. Overview of the dividing bacterium shown in Figure 7, dashed box 1. The bacterium is in close proximity to the plasma membrane (**P**). A higher maginfication (**1B**) it is shown the polymerized actin (**A**) around the bacteria. The dashed box in **1B** shows a zoom of the dense structure of the actin network (**A**), which excludes bigger material from the cytoplasm such as ribosomes. **2.** Overview of the bacterium shown with an arrow head in **Figure 7H** and **7G**, dashed box 2. The bacterium is present in a vacuole (**V**). Panel **2C** presents a zoom of an area of the vacuole where its limiting membrane (arrow) is perpendicular to the section and clearly visible. **3.** Group of bacteria shown in Figure 7, dashed box 3 devoid of actin label. In contrast to **Figure 8.1** the bacteria are present in the cytoplasm and there is no coat of polymerized actin visible. Instead, bacteria are surrounded by cytoplasm and ribosomes (R) can be seen in close vicinity to the bateria. Scale Bars: 1 *μ*m (**1A, 2A** and **3A**), 500 nm (**1B, 2B** and **3B**) and 200 *μ*m **(1C, 2C** and **3C**).