Internalization assays for Listeria monocytogenes

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Summary

Listeria monocytogenes is a model intracellular pathogen that can invade the cytoplasm of host mammalian cells. Cellular invasion can be measured using standard techniques such as the classical gentamicin protection assay, based on the quantification of colony forming units from lysates of infected cells. In addition, there are methods based on immunofluorescence microscopy which allow for assaying invasion in a medium- to high-throughput manner. In the following sections we detail two different assays that can be used alone or in combination to quantify the internalization of L. monocytogenes in host cells.

Key Words

Cell invasion, invasion assay, colony forming unit, fluorescence microscopy, medium throughput.

1. Introduction

Listeria monocytogenes is a facultative intracellular Gram-positive bacterium which invades a broad range of host cells by actin- and clathrin-dependent mechanisms triggered either through interaction of the bacterial invasion Internalin (InlA) with cellular E-cadherin, and/or InlB with the hepatocyte growth factor receptor Met (1-4). In epithelial cells, the lytic activity of the pore-forming toxin listeriolysin O (LLO) allows L. monocytogenes disruption of the vacuole in which the bacterium is entrapped after invasion and favors bacterial translocation to the host cell cytoplasm. Actin polymerization at the surface of cytoplasmic L. monocytogenes initiated by the bacterial membrane protein ActA leads to the formation of cytoskeletal structures known as 'actin comet tails' which provide a motile force that enable bacteria to move throughout the cytoplasm of primary infected cells and to spread to neighboring cells (1). In secondary infected cells, L. monocytogenes is initially located in a double-membrane vacuole that is lysed by the cooperative activities of the bacterial phospholipases PlcA and PlcB together with LLO, allowing spreading bacteria to start a new infection cycle (Fig. 1).

Different methods have been developed and improved over time to monitor the invasion of host cells by L. monocytogenes (5). A common standard assay that is used for quantifying bacterial entry, intracellular survival and replication is the gentamicin protection assay (6): in that assay, cells are infected with bacteria for a given time, followed by treatment with the non-cell permeable antibiotic gentamicin to kill extracellular L. monocytogenes; host cells are then lysed, lysates serially diluted and plated on agar plates. Quantification of bacterial colony forming units (CFUs) allows determination of the fraction of invasive L. monocytogenes that were present inside host cells at the time of gentamicin addition and were therefore protected from the antibiotic treatment. The use of different incubation times after gentamicin addition allows the system to provide additional information about the entry efficiency or the ability of bacteria to survive and/or replicate within infected cells. Microscopic assessment of intracellular versus total bacterial populations is another method to specifically assay the entry step of L. monocytogenes into host cells (7). This technique allows for a more sensitive measurement of bacterial invasion after very short internalization times since it does not require the use of gentamicin treatment after infection; moreover, this method is more robust towards cell number variation. In this chapter we will focus on the use of these two different assays in a medium-throughput manner, allowing the screening of several perturbations simultaneously.

2. Materials

All assays are optimized for HeLa cells which are only permissive for the InlB/Met dependent entry pathway. Cells are grown using standard cell culture procedures in the complete absence of antibiotics (to avoid residual bacterial killing by endocytosed antibiotics). Gentamicin protection assays are suited to investigate bacterial invasion using all strains from L. monocytogenes; as an example, we have used the strain EGDe in our experiments. Infections for microscopy-based assays in HeLa cells require more invasive strains such as the L. monocytogenes EGDe-PrfA* strain used in our protocols, which harbors a point mutation in PrfA, the major transcriptional regulator of L. monocytogenes virulence genes, rendering this regulator constitutively active and therefore leading to an increase in the expression levels of the invasion protein InlB (see Note 1).

2.1 Gentamicin protection assay

1. Cell preservation medium: 50% Dulbecco's Modified Eagle Medium (DMEM), 40% heat inactivated fetal calf serum (FCS), 10% DMSO.
2. HeLa cells 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. Infection medium: DMEM supplemented with 1% heat inactivated FCS.
5. Phosphate buffered saline (PBS).
6. Trypsin/EDTA solution.
7. L. monocytogenes strain EGDe (our own laboratory collection): kept in frozen glycerol stocks at -80°C.
8. L. monocytogenes culture medium: brain-heart infusion (BHI) as liquid medium or in agar plates.
9. 96-well cell culture plates.
10. Gentamicin.
11. Distilled water.

2.2 Differential bacterial staining assay

1. Standard cell culture medium: DMEM, 10% heat inactivated FCS.
2. L. monocytogenes strain EGDe.PrfA*-Green Fluorescent Protein (GFP).
3. Infection medium: DMEM supplemented with 1% heat inactivated FCS.

4. Listeria culture medium: BHI as a liquid medium or in form of agar plates.
5. Dark 96-well cell culture plates for cell microscopy.
6. Fixation solution: PBS, 4% para-formaldehyde (PFA).
7. PBS supplemented with 1% bovine serum albumin (BSA).
8. PBS supplemented with 1% BSA and 0.05% saponin.
9. Anti-L. monocytogenes antibody.
10. Fluorescently-labeled secondary antibodies.
11. DAPI or Hoechst, 1 mg / ml.
12. Fluorescently labeled phalloidin.
13. Fluorescence microscope equipped with a 10x or 20x objective and a screening module.
14. Multichannel pipette.

3. Methods

3.1 Gentamicin protection assay

We present the assay in a 96-well plate format, but it can be easily down-scaled to a 24-well plate format if required.

1. At least three days before the infection and starting from a frozen glycerol stock, streak L. monocytogenes strain EGDe on a BHI agar plate and grow for 48 h at 37°C: individual bacterial colonies should appear on the plate which can then be stored at 4°C for one month (while stored, seal the plate with parafilm to avoid agar desiccation).
2. At least two days before the infection, thaw HeLa cells by re-suspending a frozen cellular aliquot in 10 ml of cold standard cell culture medium and centrifuge during 5 min at 200 x g in a 15 ml polystyrene tube. Discard the supernatant, re-suspend the cellular pellet in 12 ml of warm standard cell culture medium, add the cells to a 75 cm^2 flask and transfer to a cell incubator with a humidified 5% CO_2 – containing atmosphere at 37°C.
3. The day before infection, trypsinize a flask of confluent HeLa cells, prepare a cell suspension of 100,000 cells per ml in standard cell culture medium and plate 100 µl of this suspension per well in a 96-well cell culture plate (see Notes 2 and 3). Do not use the outer wells of the plate for seeding cells and fill them instead with PBS or cell culture medium (see Note 4).
4. Move the plate quickly back and forth to uniformly distribute cells and let the cells settle down for 10 min at room temperature (see Note 5).

5. Transfer the plate to an incubator with a humidified 5% CO_2 – containing atmosphere at 37°C where cells will be allowed to attach and spread overnight.
6. The day before the infection prepare a liquid culture of L. monocytogenes by inoculating a bacterial colony from the BHI agar plate to 5 ml of BHI liquid medium contained within a 15 ml polystyrene tube. Let the culture grow overnight at 37°C in a shaker.
7. The actual day of the infection, take 1 ml of the overnight L. monocytogenes culture, centrifuge during 2 min at 10,600 x g in a table-top centrifuge, discard the supernatant (containing the secreted cytotoxin listeriolysin O) and re-suspend the pellet in 1 ml of PBS (see Note 6).
8. Repeat the washing steps 3 more times.
9. Read the bacterial optical density at 600 nm and estimate the number of bacteria (OD=1 is equivalent of $1x10^9$ bacteria/ml).
10. Prepare the inoculum by diluting bacteria in the appropriate volume of infection medium (see Notes 7 and 8).
11. Aspirate the medium from HeLa cells and add 100 µl of the inoculum to each well (see Note 9).
12. Transfer the inoculated plate to an incubator with a humidified 5% CO_2 atmosphere at 37°C and allow L. monocytogenes to invade the cells for 1 h.
13. During this incubation time, plate a dilution of the inoculum to precisely establish the number of bacteria that were used for infection. For this, using four wells from a 96-well plate row, put 90 µl of distilled water in each well and make a first 1:10 dilution of the inoculum by adding 10 µl of the inoculum to the first well of the row; homogenize and take 10 µl of this dilution that will be diluted in 90 µl of distilled water in the second well of the row; repeat two additional 1:10 dilutions and plate 10 µl of dilutions 2, 3 and 4 on the top of a 10 cm BHI agar plate in triplicate using a multichannel pipette. Plate 6 lysates per 10 cm plate by pipetting the drops on top of the plate, then tilt the plate to let them run down to make lines of lysate (Fig. 2) (see Note 10).
14. After the cellular infection has been completed for 1 h, wash the plate once with 100 µl per well of pre-warmed standard cell culture medium supplemented with 40 µg/ml gentamicin, and replace by 100 µl of the same gentamicin-containing medium.
15. Transfer the 96-well plate back to the incubator and keep it at 37°C in 5% CO_2 humidified atmosphere for another hour for assaying entry (if intracellular survival or replication is assayed, start the experiment by preparing and infecting two identical 96-well plates, of which one should be kept in the incubator for 1 h and the other one for 4 h after addition of gentamicin) (see Note 11).

16. After the killing of the extracellular bacteria is completed by the gentamicin treatment, wash the cells once with 100 µl of standard cell culture medium without gentamicin per well, remove the medium and lyse the cells by adding 100 µl of distilled water per well.
17. To completely disrupt the HeLa cells, pipette the water 5 to 10 times up and down using a multichannel pipette and scrape the cells by pressing the pipette tips on the well bottom while pipetting (see Note 12).
18. Make a 1:10 dilution in a new 96 well plate by adding 10 µl lysate to 90 µl distilled water per well. Dilute the lysates two more times to obtain 1:100 and 1:1000 dilutions.
19. Plate 10 µl of each lysate and dilution on a 10 cm BHI agar plate: 6 lysates can be plated on one plate as described in step 13 and Fig. 2.
20. Let the lysates dry by opening the BHI agar plates under the microbiological hood for a few minutes.
21. Put the lid back on the BHI agar plates and transfer them to an incubator at 37°C to let bacterial colonies grow overnight.
22. The day after the infection, count colonies of each row for the dilution where colonies are well separated from each other (Fig. 3) and calculate entry as the ratio of colony forming units (CFU) of lysates and inoculum. For quantifying intracellular survival or replication, calculate then the ratio of the late- and early time points (see Note 13).

3.2 Differential bacterial staining

As with the previous protocol, we present this particular assay in a 96-well plate format with the goal of performing medium- to high-throughput assays with image acquisition using a microscope equipped with an automatic plate-reading system. The procedure can be up-scaled to 384-well plates for the study of small interfering RNA libraries (see Note 3), or down-scaled to a 24-well plate format in which coverslips are added in each well for individual treatment of each condition.

1. HeLa cells and L. monocytogenes EGDe.PrfA*-GFP are grown as described in Step 1 and Step 2 of section 3.1of the Gentamicin protection assay 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 Step 3 to Step 5 of section 3.1, plating the cells in dark microscopy 96 well plates (instead of transparent plates).

3. Prepare the L. monocytogenes EGDe.PrfA*-GFP strain as described in section 3.1, Steps 6 to 10.
4. Infect cells as described in section 3.1, Step 11.
5. Centrifuge the plate for 5 min at 200 x g at room temperature to bring L. monocytogenes in close contact to HeLa cells in order to synchronize infection.
6. Transfer the plate to an incubator with a humidified 5% CO_2 atmosphere at 37°C and let bacteria enter cells for 30 min (see Note 14).
7. Aspirate infection medium and wash cells once with 100 µl of standard cell culture medium per well.
8. Fix cells by adding 100 µl of PBS supplemented with 4% PFA to each well and incubate at 4°C for 15 min.
9. Remove the fixative, wash cells 3 times with 100 µl PBS per well and keep cells in 100 µl PBS per well.
10. Prepare the primary antibody solution by diluting a rabbit-derived anti-L. monocytogenes serum 1:300 in PBS supplemented with 1% bovine serum albumin and add 30 µl of primary antibody to each well after removing the PBS.
11. Incubate for 15 min at room temperature to label extracellular L. monocytogenes.
12. Discard the primary antibody solution and wash six times with 100 µl of PBS per well.
13. Dilute the secondary AlexaFluor 546-coupled anti-rabbi antibody (1:300) in PBS and add 30 µl of this secondary antibody solution to each well after removing the PBS.
14. Incubate for 30 min at room temperature in the dark to protect the fluorescent probe.
15. Discard the secondary antibody solution and wash six times with 100 µl of PBS per well.
16. Dilute DAPI solution (1:1500) and phalloidin Dy 647 (1:150) in PBS, 0.05% saponin and add 30 µl of this staining solution to each well after removing the PBS.
17. Incubate for 30 min at room temperature in the dark to protect the fluorescent probes.
18. Discard the secondary staining solution, wash four times with 100 µl of PBS per well. And keep the cells in 100 µl of PBS per well.
19. Acquire images in the 350 nm (DAPI), 488 nm (total L. monocytogenes), 546 nm (extra-cellular L. monocytogenes) and 647 nm (actin) channels using a 20x objective mounted on an automated microscope.
20. Entry can be quantified as ratio of intracellular L. monocytogenes (GFP-positive bacteria) minus extracellular L. monocytogenes (AlexaFluor 546-positive bacteria) divided by total cell associated bacteria (see Note 15). In addition, the total number of bacteria per cell can give indications on the ability of bacteria to bind to cells. Bacteria can be counted manually, or by using appropriate image analysis software.

4. Notes

1. The protocols that we present in this chapter are optimized for HeLa cells (human uterus) which are invaded by L. monocytogenes only via the InlB/Met pathway. Polarized epithelial cells such as Caco-2 (human intestine), LoVo (human colon) and Jeg3 or Bewo (human trophoblasts) can be additionally invaded via the InlA/E-cadherin pathway and are therefore more sensitive to L. monocytogenes infection; our protocols can be applied to these cell lines but the multiplicity of infection (MOI) should be reduced to account for the greater number of bacteria that will invade these host cells. These cell lines are particularly relevant for the microscopical study of bacterial internalization using poorly invasive strains of L. monocytogenes such as the EGDe strain.
2. The number of plated wells will vary according to the number of different conditions that will be analyzed: we typically seed cells in triplicate (three independent wells) for each condition.
3. All assays described here can be used in combination with small interfering RNA (siRNA)-mediated knock down of host cell factors to study the involvement of a given host cell protein in the L. monocytogenes infection process. If cells are transfected with siRNA 72 h before infection, the cell number plated per well should be reduced to 5400 cells for 96 well plates (or to 600 cells for 384 well plates). Special attention should be made to ensure that cell death due to siRNA treatment is not perturbing the final cell number counts.
4. Outer wells are exposed to more temperature variations and evaporation than inner wells and results proceeding from cells seeded in these wells differ consistently from those of cells seeded in more central wells. We therefore avoid using the outer wells for cell seeding and we fill them instead with a solution that will serve as a temperature- and evaporation buffer for inner wells.
5. Differences in infection levels dependent on the well position on a plate can cause important artifacts. There are several possibilities to reduce these effects: for example, letting cells settle down after seeding for 10 min at room temperature may also reduce plate effects. Keeping plates on aluminum blocks in the incubator ensures more equal temperature distribution across the plate and sealing plates with parafilm prevents evaporation of medium from edge- and especially corner wells. In addition, a random distribution of controls and perturbation conditions on the plate can minimize a systematic accumulation of plate effects causing artifacts.
6. LLO is a cytotoxin that can induce cell death especially if bacteria are not well washed before infection, if the MOI is too high or if there is too much time passing between washing bacteria and infecting cells. LLO can especially cause problems when highly invasive strains like

EGDe.PrfA* are used. Therefore, it is important to check under a microscope if cells detach during the experiment. This is especially important for classical gentamicin protection assay since there the total number of bacteria in the whole cell lysate is used as the measure for infection, meaning that differences in cell number can significantly influence the outcome of the experiment.

7. The specific MOI of the inoculum needs to be adjusted according to the L. monocytogenes strain that is examined in the assay: when using the poorly invasive strain EGDe we prepare an inoculum containing between $1x10^7$ and $2x10^7$ bacteria per ml (the final MOI = 50 to 100 estimated for 20,000 cells per well). When using more invasive L. monocytogenes strains such as EGDe.PrfA* the MOI can be decreased by a factor of 5.
8. We use usually 1% FCS in infection media, however in some cases residual FCS can influence cellular signaling in a way that it interferes with the effect that one would like to assay. In these cases it is recommended not to use FCS in the infection medium.
9. Since the results will strictly depend on the number of cells present in individual wells, it is critical not to increase variation by removing cells throughout the different washing steps that the experiment requires. Therefore, addition of new medium should be performed gently to avoid removal of attached cells by sheer mechanical forces.
10. In our experience, the drops of lysate run straight down on tilted BHI agar plates when the agar is fresh with an even surface, meaning that the agar should not be too dry on the surface. We noticed that some dilutions run down better than others. Running down of the lysates can be improved by slightly shaking the tilted plate vertically against the bench. Nevertheless, we recommend preparing spare agar plates for the experiment in case certain plates need to be repeated.
11. For the study of survival/replication of L. monocytogenes within host cells, the incubation time after addition of gentamicin can be varied between 1 and 5 h after infection (after that length of time, in addition to entry into primary infected cells there will be bacterial cell-to-cell spread to neighboring cells). For a more precise quantification of replication, an individual plate can actually be used to screen each individual time point after gentamicin addition.
12. HeLa cells are easily disrupted using the proposed protocol; however, other cell lines could require additional rounds of pipetting for complete disruption and microscopical verification of cell lysis using transmitted white light is suggested.
13. Example of calculations for a theoretical experiment:
    CFU (input) dilution 4: 10
    CFU (output 1+1h) dilution 2: 12

CFU (output 1+4h) dilution 3: 9

$$\%\ invasion\ (of\ inoculum) = 100 \times \frac{CFU\ (output) \times dilution(output)}{CFU\ (input) \times dilution(input)}$$

$$\%\ invasion\ after\ 1 + 1h = 100 \times \frac{12 \times 100}{10 \times 10\ 000} = 1.2$$

$$\%\ invasion\ after\ 1 + 4h = 100 \times \frac{9 \times 1000}{10 \times 10\ 000} = 9$$

$$fold\ change\ of\ intracellular\ bacteria\ within\ 3h = \frac{\%\ invasion\ (1 + 4h)}{\%\ invasion\ (1 + 1h)}$$

$$fold\ change\ of\ intracellular\ bacteria\ within\ 3h = \frac{9}{1.2} = 7.5$$

14. For microscopical assessment of invasion, cells can be infected for time periods as short as 5 min or as long as 4 h (the actual time limits will depend on the specific cell lines and the bacterial strains used). A time course for entry can be done using this technique.
15. For differential bacteria staining, it is possible to use non-GFP expressing bacteria: do a first staining of extracellular bacteria without saponin in the staining solution, wash thoroughly 3 to 4 times with PBS, 1% BSA followed by a second staining with primary and secondary antibody against total L. monocytogenes, using staining solution containing 0.05% saponin for cell permeabilization. We do this for small scale experiments on coverslips, however for large scale experiments the use of GFP expression to visualize total bacteria can simplify the experiment and make it more robust towards cross-staining between total and extracellular bacteria.

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Figure Legends

Figure 1. L. monocytogenes intracellular cycle. L. monocytogenes promotes entry into target cells through interaction of the bacterial surface proteins InlA and InlB with their cellular receptors E-cadherin and Met, respectively. Rupture of the bacterial internalization vacuole is subsequently achieved by the secreted toxin LLO. Within the host cell cytoplasm, L. monocytogenes proliferates and induces actin polymerization by the surface protein ActA, favoring bacterial invasion of neighboring cells. The L. monocytogenes-containing vacuole in secondary infected cells is disrupted by the combined activity of LLO and the two bacterial phospholipases PlcA and PlcB.

Figure 2. Pipetting L. monocytogenes CFUs for the Gentamicin Protection Assay. A. After cellular lysis with distilled water, 10 µl containing re-suspended L. monocytogenes CFUs from six independent wells (using a 96-well plate) are aspirated using a multichannel pipette. B. The 10 µl are deposited at the border of 10 cm BHI agar plate. C. The agar plate is tilted to allow the water drops containing the CFUs to run along the plate. D. The drops are allowed to make a water path along the agar, after which the plate is allowed to dry and incubated at 37°C.

Figure 3. Fully grown L. monocytogenes CFUs. 24 h after plating and growth at 37°C, the individual L. monocytogenes CFUs can be counted to estimate bacterial entry into host cells.