

Video Article

# Purification of Pathogen Vacuoles from *Legionella*-infected Phagocytes

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## Abstract

The opportunistic pathogen *Legionella pneumophila* is an amoeba-resistant bacterium, which also replicates in alveolar macrophages thus causing the severe pneumonia "Legionnaires' disease"<sup>1</sup>. In protozoan and mammalian phagocytes, *L. pneumophila* employs a conserved mechanism to form a specific, replication-permissive compartment, the "Legionella-containing vacuole" (LCV). LCV formation requires the bacterial lcm/Dot type IV secretion system (T4SS), which translocates as many as 275 "effector" proteins into host cells. The effectors manipulate host proteins as well as lipids and communicate with secretory, endosomal and mitochondrial organelles<sup>2-4</sup>.

The formation of LCVs represents a complex, robust and redundant process, which is difficult to grasp in a reductionist manner. An integrative approach is required to comprehensively understand LCV formation, including a global analysis of pathogen-host factor interactions and their temporal and spatial dynamics. As a first step towards this goal, intact LCVs are purified and analyzed by proteomics and lipidomics.

The composition and formation of pathogen-containing vacuoles has been investigated by proteomic analysis using liquid chromatography or 2-D gel electrophoresis coupled to mass-spectrometry. Vacuoles isolated from either the social soil amoeba *Dictyostelium discoideum* or mammalian phagocytes harboured *Leishmania*<sup>5</sup>, *Listeria*<sup>6</sup>, *Mycobacterium*<sup>7</sup>, *Rhodococcus*<sup>8</sup>, *Salmonella*<sup>9</sup> or *Legionella* spp.<sup>10</sup>. However, the purification protocols employed in these studies are time-consuming and tedious, as they require e.g. electron microscopy to analyse LCV morphology, integrity and purity. Additionally, these protocols do not exploit specific features of the pathogen vacuole for enrichment.

The method presented here overcomes these limitations by employing *D. discoideum* producing a fluorescent LCV marker and by targeting the bacterial effector protein SidC, which selectively anchors to the LCV membrane by binding to phosphatidylinositol 4-phosphate (PtdIns(4)P)<sup>3,11</sup>. LCVs are enriched in a first step by immuno-magnetic separation using an affinity-purified primary antibody against SidC and a secondary antibody coupled to magnetic beads, followed in a second step by a classical Histodenz density gradient centrifugation<sup>12,13</sup> (**Fig. 1**).

A proteome study of isolated LCVs from *D. discoideum* revealed more than 560 host cell proteins, including proteins associated with phagocytic vesicles, mitochondria, ER and Golgi, as well as several GTPases, which have not been implicated in LCV formation before<sup>13</sup>. LCVs enriched and purified with the protocol outlined here can be further analyzed by microscopy (immunofluorescence, electron microscopy), biochemical methods (Western blot) and proteomic or lipidomic approaches.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/4118/>

## Protocol

### 1. Preparations for LCV Isolation

1. Streak out *Legionella pneumophila* producing DsRed-Express<sup>13,14</sup> from glycerol stocks on CYE agar plates with 5 µg/mL chloramphenicol (Cam) four days before LCV isolation. Incubate the bacteria at 37 °C.
2. Seed out  $1 \times 10^7$  *D. discoideum* producing GFP-calnexin or RAW264.7 murine macrophages in 75 cm<sup>2</sup> tissue culture flasks one day prior infection. Use 10 ml HL5 medium with 20 µg/mL G418 for *D. discoideum* and incubate the amoebae at 23 °C. For RAW264.7 macrophages use 10 ml RPMI 1640 medium supplemented with 10% FCS (heat inactivated) and 1% glutamine, and grow the cells at 37 °C and 5% CO<sub>2</sub>. Use at least three 75 cm<sup>2</sup> flasks per infection and sample (minimum  $6 \times 10^7$  cells).
3. Inoculate an overnight culture with *L. pneumophila* from the CYE plate. Take a 15 mL test tube with 3 mL AYE medium and 5 µg/mL Cam. Inoculate with 100 µl of a bacterial suspension to yield an OD<sub>600nm</sub> of 0.1. Incubate the overnight culture on an overhead rotation wheel at 37 °C for 21-22 hr.

## 2. LCV Isolation

1. Change the medium of *D. discoideum* cells to remove the antibiotic, which would interfere with the following infection.
2. Measure the OD of the overnight culture. Bacteria should have reached their peak infectivity at an  $OD_{600nm}$  of  $\geq 3$ , which corresponds to  $2 \times 10^9$  bacteria/mL.
3. Infect the cells by adding approximately 500  $\mu$ l of the *L. pneumophila* overnight culture to the cells growing in 10 ml HL5 medium (*D. discoideum*) or 10 ml RPMI 1640 (macrophages). This corresponds to a multiplicity of infection (MOI) of 50. Subsequently, the infection is synchronized by centrifugation of the bacteria onto the cells at  $500 \times g$  for 10 min. Centrifugation is followed by an incubation of the *D. discoideum* cells at 25 °C and the RAW264.7 macrophages at 37 °C and 5% CO<sub>2</sub>, respectively. The infection time of 1 hr includes the centrifugation step.
4. After infection remove the medium and wash the cells once to remove extracellular bacteria. Use ice-cold SorC buffer for *D. discoideum* and ice-cold PBS for RAW264.7 macrophages. Add 3 mL HS buffer, supplemented with protease inhibitors (Roche) to each flask and collect the cells by using a cell scraper. Pool the corresponding samples in a 15 mL test tube.
5. For homogenization of the sample use 3 mL plastic Luer-Lock syringes and the stainless steel ball homogenizer. Make sure that you work on ice. Before starting, wash the ball homogenizer with distilled water, to avoid any detergent contamination and flush it with ice cold HS buffer to get rid of air bubbles. Use the 8  $\mu$ m clearance ball.
6. Fill the first 3 mL into the syringe and mount it on the homogenizer. Press the sample back and forth nine times through the homogenizer. Exchange the syringes afterwards to avoid contamination with not-homogenized material. Collect and pool the homogenized sample in a 15 mL test tube and take a 150  $\mu$ l sample for microscopic analysis. Before proceeding with a different sample dismantle and wash the ball homogenizer.
7. Block the homogenate with 2% CS or FCS for 30 min on a shaker on ice or on an overhead-spinning wheel (10-20 rpm) at 4 °C.
8. After blocking use an affinity-purified primary antibody against SidC (dilution 1:3000) or against any other bacterial marker exclusively binding to the LCV membrane. Vortex the antibody solution before adding it to the homogenate. Incubate the sample for 1 hr on a shaker on ice or on an overhead-spinning wheel (10-20 rpm) at 4 °C.
9. In the meantime, prepare the Histodenz gradient. Use a 15 mL test tube per gradient and three 75 cm<sup>2</sup> flasks of one sample. First, add 5.75 mL of 35% Histodenz to the tube, and second, carefully add 5.75 mL of 10% Histodenz solution on top. Afterwards carefully lay the tubes down horizontally for 1 hr.
10. After incubation of the homogenate with the blocking reagent and the primary antibody, centrifuge at  $600 \times g$  for 15 min at 4 °C to pellet the sample. Discard the supernatant and resuspend the pellet in 1.5 mL HS buffer. Transfer the samples to a fresh 15 mL test tube and take a 40  $\mu$ l sample for microscopic analysis later on.
11. Incubate the pellet with the secondary antibody - MACS goat anti-rabbit IgG micro beads (dilution 1:25) - for 30 min on a shaker at 4 °C. In the meantime, put the columns on the MACS magnetic holder and equilibrate them with 0.5 mL HS buffer.
12. Subsequently, apply the samples to the column. Collect 150  $\mu$ l of the flow-through for subsequent microscopic analysis. Wash the column three times with 0.5 mL HS buffer.
13. Remove the columns from the magnet and eluate the LCVs with 0.5 mL HS buffer by immediate squirting. Take a 20  $\mu$ l sample for microscopic analysis. The purification step by immuno-magnetic separation is expected to yield per  $1 \times 10^7$  infected *D. discoideum* cells approximately  $1 \times 10^6$  intact LCVs, which are about sevenfold enriched in the eluate compared with the flow-through<sup>13</sup>.
14. Put the Histodenz gradient tubes back to a vertical position and carefully load the eluate on top. Centrifuge the tubes at  $3350 \times g$  for 1 hr at 4 °C.
15. After centrifugation take 1.5 mL fractions starting from the bottom of the test tube by using a long glass Pasteur pipette. Altogether collect eight fractions, each from the bottom of the tube. No visible layers are observed in the continuous Histodenz gradient. The highest yield of LCVs is typically expected in fraction 4 at a ratio of  $\sim 2 \times 10^5$  intact LCVs per  $1 \times 10^7$  infected amoebae<sup>13</sup>. Minor quantities of LCVs are also present in fractions 3 and 5, respectively. Thus, the overall yield of purified LCVs is  $\sim 1 \times 10^6$  intact LCVs per  $6 \times 10^7$  infected *D. discoideum* cells<sup>13</sup>. The recovery of LCVs can be performed at room temperature (RT), and the LCVs appear stable for several hours.

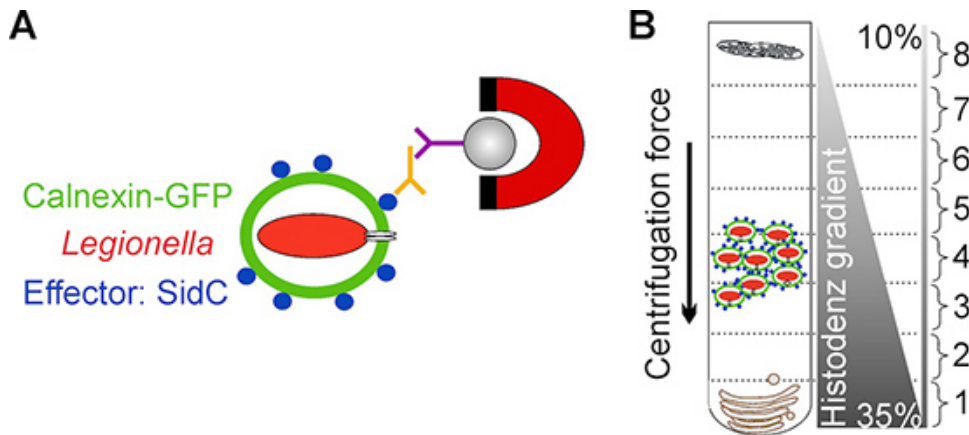
## 3. Microscopic Analysis of the Collected Samples

1. Prepare a 24-well flat-bottom tissue culture plate with poly-L-lysine coated coverslips.
2. Pipette every sample taken during the LCV purification plus 150  $\mu$ l of each density gradient fraction into the 24-well plate. Add 0.5 mL HS buffer to each well to dilute the high Histodenz concentration. Afterwards, centrifuge the plate at  $600 \times g$  for 10 min at 4 °C. Carefully remove the supernatant and fix the samples with 4% paraformaldehyde (PFA) for 20 min at RT. After fixation wash the samples twice. Use SorC for *D. discoideum* and PBS for RAW264.7 macrophages.
3.
  - a. Mount the samples from GFP-calnexin expressing *D. discoideum*, taken at different steps during LCV isolation, directly on glass-slides using e.g. Vectashield.
  - b. Incubate the samples from RAW264.7 macrophages, taken at different steps during LCV isolation, with blocking solution (1% BSA in PBS) for 10 min at RT. Use an anti-SidC primary antibody to stain the LCVs and incubate for 1h in a wet chamber at RT (affinity-purified rabbit anti-SidC; 1:1000 in blocking buffer). Subsequently, wash the samples three times with PBS. Incubate with a secondary antibody (e.g. anti-rabbit Cy5; 1:200 in blocking solution) for 30 min in a wet and dark chamber at RT. Finally, wash the samples three times with PBS and mount them on glass-slides using e.g. Vectashield.
4. Morphology, integrity and quantity of the LCVs isolated from GFP-calnexin expressing *D. discoideum* or from RAW264.7 macrophages are analyzed by a fluorescence microscope equipped with the adequate filters.

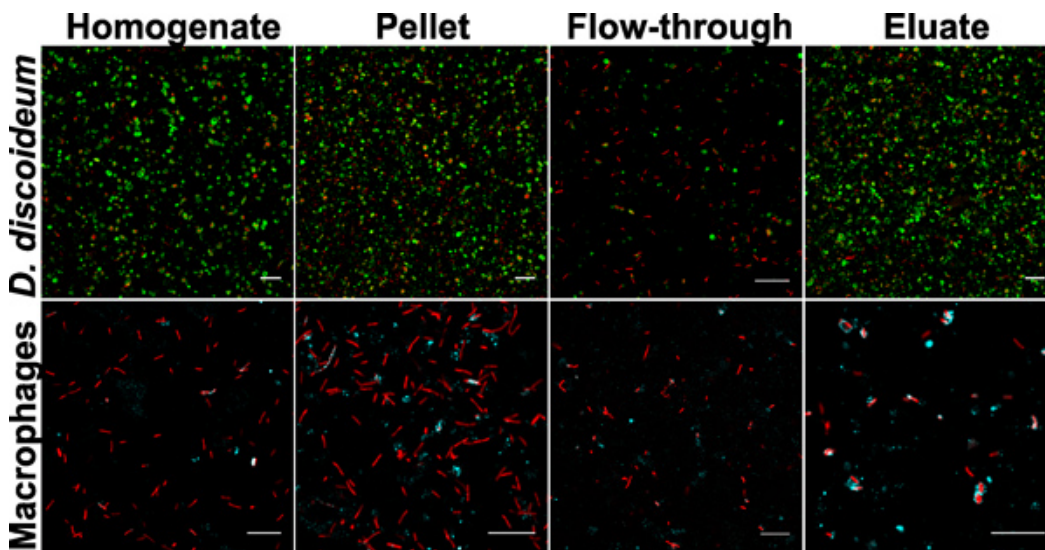
## 4. Representative Results

The quality and yield of the LCV purification by immuno-affinity separation and density gradient centrifugation can be followed by fluorescence microscopy (Fig. 1). An overview of samples collected during the LCV isolation from *D. discoideum* or macrophages is shown (Fig. 2). The homogenate of *L. pneumophila*-infected phagocytes shows intact LCVs, but also a lot of cell debris and extracellular bacteria. After pelleting the

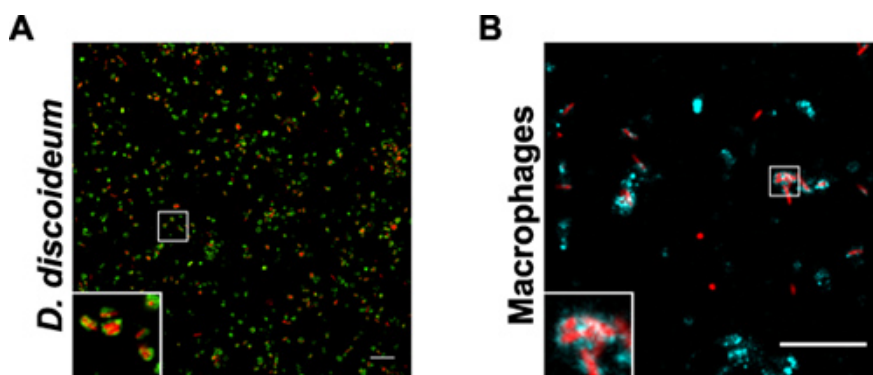
sample, the image is similar to the homogenate, sometimes a bit denser. Since intact LCVs should stick to the column, the flow-through contains mostly extracellular bacteria and cell debris. After eluting the sample from the column, the eluate contains intact LCVs in large amounts. In our hands, the LCV purification appears to be more effective for *D. discoideum* than for macrophages. In *D. discoideum* the pathogen vacuoles appear rounder and the yield of isolated LCVs is more than 10 times higher compared with macrophages (Fig. 3). In intact macrophages LCVs stained with different antibodies also appear more irregularly shaped.



**Figure 1.** Schematic overview of LCV isolation. A) Isolation of LCVs by immuno-magnetic separation using an affinity-purified antibody against the bacterial effector protein SidC, localizing exclusively to the LCV membrane. A secondary MACS micro bead-coupled antibody and a magnet are used to isolate LCVs from cell debris. B) After immuno-magnetic separation LCVs are further purified by Histodenz density gradient centrifugation. LCVs are enriched in fraction 4.



**Figure 2.** Samples collected during LCV purification. Images from homogenate, pellet, flow-through and eluate from *D. discoideum* or macrophages are depicted. The samples show *L. pneumophila* producing DsRed-Express (red) in *D. discoideum* producing GFP-calnexin signal (green, upper panel) or in macrophages stained with an anti-SidC antibody (cyan, lower panel). Bars, 10 µm.



**Figure 3.** Purified LCVs from *D. discoideum* or macrophages. The samples show fraction 4 after Histodenz density gradient centrifugation of *L. pneumophila* producing DsRed-Express (red) in (A) *D. discoideum* producing GFP-calnexin signal (green) or (B) in macrophages stained with an anti-SidC antibody (cyan). Bars, 10  $\mu$ m.

## Discussion

In contrast to previously published methods, this protocol is based on two steps, first the separation of LCVs by an immuno-magnetic approach, and second, the purification of LCVs by density gradient centrifugation. The LCV isolation can be easily followed by fluorescence microscopy, when either fluorescently labeled bacteria and GFP-producing cells or antibody staining is used. The protocol described here is a simple, straight-forward method, which results in the enrichment of LCVs with high purity. Importantly, the protease inhibitor in the homogenization buffer is required for RAW264.7 macrophages, but optional for *D. discoideum*<sup>13</sup>.

In principle, the protocol can be adopted for other pathogens or host cells, as long as a specific, selectively localizing marker is present, which is needed for the separation step. Ideally, the distinct marker is of bacterial origin, transported to the vacuole membrane, and selectively localizing there. Targeting a host cell marker likely results in the undesired co-purification of the pathogen vacuole with other host cell compartments.

Once a distinctive marker of the pathogen vacuole is identified, a suitable polyclonal or monoclonal antibody has to be raised. For every primary antibody the concentration and the blocking reagent should be optimized, as blocking reagents other than FCS could be more appropriate (e.g. BSA, de-lipidated milk, other commercially available blocking reagents).

Another critical point is the infectivity of the pathogen. The bacteria used for infection should be in their most infective stage. *L. pneumophila*, e.g., needs to be grown to the post-exponential/early stationary growth phase in liquid culture. Under these conditions, the bacteria are also morphologically uniform (~2 x 0.5  $\mu$ m), as opposed to bacteria grown on CYE agar plates. Furthermore, antibiotics possibly used in the cell culture medium must be removed prior to an infection to avoid low infectivity and harm to the bacteria. To avoid a negative effect on the uptake efficiency, the phagocytes should have reached a confluency of approximately 80% (not more) at the time of infection. Finally, although an incubation period of 1 hr is the standard infection time for *L. pneumophila*, yielding a rather homogenous and robust population of LCVs, other time points can be chosen for proteomic or biochemical studies on LCV formation<sup>13</sup>.

Once the above method is established for a given pathogen-host cell pair, it is also possible to test bacterial mutant strains and/or different host cells, including *D. discoideum* defined deletion mutants. In general, pathogen vacuoles purified with this protocol can be analyzed by different methods, including microscopy (fluorescence and electron microscopy), biochemical assays (Western blot), as well as proteomics and lipidomics. For the latter methods, it is advisable to coordinate with the person in charge of the proteomics/ lipidomics analysis the buffer conditions, sample amounts and downstream processing of purified pathogen vacuoles.

## Disclosures

No conflicts of interest declared.

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