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1 **Sorting of phagocytic cells infected with *Legionella***
2 ***pneumophila***

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11 **Running title:** FACS sorting of *Legionella* infected cells
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Abstract

The ability of *Legionella pneumophila* to colonize host cells and to form a replicative vacuole depends on its ability to counteract the host cell response by secreting more than 300 effectors. The host cell responds to this bacterial invasion by counteracting the pathogens with extensive intracellular signaling. When studying *L. pneumophila* infection *in vitro* only a small proportion of the cell lines or primary cells used to analyze the host response are infected, therefore the study of this mixed cell population would lead to unprecise results. In order to study the multitude of pathogen-induced phenotypic changes occurring in the host cell, the separation of infected from uninfected cells is thus a top-priority. Here we describe a highly efficient FACS derived protocol to separate cells infected with a *L. pneumophila* strain encoding a fluorescent protein. Indeed, the highly homogenous infected cell population obtained after sorting is the best possible starting point for the subsequent studies of infection-induced effects.

Key Words: *Legionella pneumophila*, phagocytic cells, fluorescent proteins, single cell sorting.

1 Introduction

The Gram-negative bacterium *Legionella pneumophila* is able to form an intracellular niche in infected cells allowing it to replicate and then to spread in the extracellular medium to re-infect new host cells (1). The formation of a vacuole and the hijacking of cellular response require a functional Type-4 Secretion System (T4SS) that allows the injection of more than 300 effectors in the host cell, which are then subverting signal transduction and vesicle trafficking pathways (2).

Cellular microbiology studies in the *Legionella* field are often the characterization of the functions of the secreted effectors, in order to understand how they interact and modify the cellular targets, as well as the mechanisms the target cell employs to modify its signaling pathways to counteract the infection. The modulation of cellular responses is a key step of the invasion strategy of *L. pneumophila* at all different stages of the *L. pneumophila* life cycle, such as during bacterial uptake, the formation of the *Legionella* containing vacuole, the survival and replication in the host cell. A wide range of techniques allows the study of the host cell responses to infection, such as the analyses of the transcriptional and proteomic response or genetic modulations (3-8). However, in order to study the regulation of host cell signaling during infection a cell population uniformly infected is needed, however when infecting eukaryotic cells (e.g. THP-1, A549, primary macrophages, *Acanthamoeba castellanii*) with *L. pneumophila* only a small proportion of the cells is indeed infected. Thus it is necessary to separate infected and uninfected cells to avoid the analyses of a mixture of two populations and to eliminate the background noise.

Fluorescence-activated cell sorting (FACS) is a widely used tool in biological research that utilizes laser-based technology to count, sort and profile a heterogeneous population of cells in a fluid mixture (**Fig. 1**). This technique allows for a rapid and highly accurate quantitation of individual cells based on differential fluorescence intensities or biological characteristics, such as size (by forward-scattered light; FSC) and internal structure (by side-scattered light; SSC). The sorting function allows high-speed, accurate separation and collection of individual cells based on user-defined flow cytometry parameters. Beyond the exceptionally accurate and analytical nature, cell sorting is non-destructive with little to no effect on cell viability or function.

This protocol describes infection assays with *L. pneumophila* strains producing fluorescent proteins: these strains are not impaired for virulence and have already been used to analyze infected host cells by flow cytometry (9) or fluorescence microscopy (10). Cells infected with fluorescent bacteria are placed in a stream of liquid which passes the focus of a laser and will be sorted from the uninfected population. We set up this method for human monocytes and amoebae, but it can be applied to any cell type.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Strains and growth media

1. We used a *L. pneumophila* strain Paris harboring a pBC-KS plasmid encoding for a GFP protein under the control of the *flaA* promoter (our unpublished data). In these conditions, the GFP protein is produced in the post-exponential (virulent) phase (see **Note 1**). *L. pneumophila* grows at 37°C and requires L-cysteine and iron.
2. CYE (charcoal yeast extract) agar plates: 10 g/L ACES, 10 g/L yeast extract in water; adjust the pH to 6.9 with KOH; add 15 g/L agar and 2 g/L charcoal powder; autoclave and let the agar solution cool down to 50°C. Add filter sterilized L-cysteine, iron pyrophosphate solutions (see **Note 2**) and antibiotics. Store the plates at 4°C.
3. AYE (ACES yeast extract) medium: 10 g/L ACES, 10 g/L yeast extract; adjust the pH to 6.9 with KOH. Filter sterilize using a 0.2 µm filter cartouche and add filter sterilized L-cysteine, iron pyrophosphate solutions (see **Note 2**) and antibiotics. Store at 4°C.
4. Antibiotics: 10 µg/ml chloramphenicol (Cam, stock: 100 mg/ml in ethanol).
5. Dulbecco's phosphate-buffered saline (D-PBS) without Ca²⁺ and Mg²⁺.
6. Spectrophotometer and plastic cuvettes to measure the OD.

2.2 *Acanthamoeba castellanii*

1. The *Acanthamoeba castellanii* strain ATCC50739 was cultured at 20°C.
2. PYG 712 medium: rich medium for *A. castellanii* growth. 2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05

mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM NaH_2PO_3 , 2.5 mM K_2HPO_3 . Sterilize the medium using a 0.2 μm filter cartouche and store at 20°C.

3. Infection buffer: PYG 712 medium without proteose peptone, glucose and yeast extract.

This minimal medium is used for intracellular growth assays.

4. Culture flasks: 25 cm^2 or 75 cm^2 ; filter screw cap; growth-enhanced treated; sterilized by radiation; free from pyrogens, DNA/RNA and DNase/RNase.

5. Cell counting chamber slides.

6. Cell incubator set at 20°C.

2.3 THP-1 macrophages

1. The THP1 cell line ATCC TIB202 is used and grown at 37°C and 5% CO_2 in humidified air in an incubator.

2. Supplement the commercially available RPMI 1640 GlutaMAX™ Supplement medium with 10% heat-inactivated fetal calf serum (FCS).

3. Infection assays were performed in RPMI 1640 GlutaMAX™ Supplement medium without FCS.

4. Culture flasks; 25 cm^2 or 75 cm^2 ; filter screw cap; growth-enhanced treated; sterilized by radiation; free from pyrogens, DNA/RNA and DNase/RNase.

5. Cell counting chamber slides.

6. Cell culture incubator

7. Centrifuge equipped with a swing rotor.

2.4 Cell Sorter

1. The cell sorter used is a FACS Aria II (Becton Dickinson) equipped with a 488 nm excitation source (*see Note 3*) and a 70 or 85 μm nozzle (*see Note 4*).

2. The FSC/SSC and fluorescence signals are acquired and quantified using the FACS DIVA software (Becton Dickinson) and FlowJo (Treestar).

3. The samples are resuspended in FACS buffer (PBS; 10 mM HEPES; 2% FCS; 2 mM EDTA) (*see Note 5*) and measured in sterile polypropylene tubes pre-coated with serum or culture medium at 37°C (*see Note 6*).

4. Infected cells might be fixed in PBS-4% paraformaldehyde solution.

3 Methods

3.1 Growth of *L. pneumophila*

1. Streak out *L. pneumophila* from frozen glycerol stocks onto CYE/Cam plates. A bacterial lawn will form after 3-4 days of incubation at 37°C.
2. The day of infection suspend a loop of bacteria grown on CYE/Cam plates in 900 µl PBS, mix thoroughly, measure the OD₆₀₀ and adjust the density of the bacterial suspension to OD₆₀₀ of 2 (*see Note 7*). The multiplicity of infection (MOI) is defined as the number of added bacteria per individual host cell, calculate the bacterial concentration of your suspension, given that an OD₆₀₀ of 2.2 corresponds to approximately 2x10⁹ bacteria/mL (*see Note 8*).

3.2 Culture of *A. castellanii* and infection assays

1. Cultivate *A. castellanii* in PYG medium at 20°C in culture flasks (25 cm² or 75 cm² depending on the number of cells needed).
2. Split the culture every week: remove PYG medium, add 10 ml of PBS, tap off the cells, and count them on a cell counting chamber slide. Expand the cells depending on the amount needed for the infection assay (*see Note 9*).
3. The day of infection seed 10⁷ cells/T75 cm² culture flasks in 10 ml of infection medium per condition. Let the cells settle and adhere to the plastic surface for 1-2 hours. Prepare one additional flask for uninfected cells.
4. Infect by adding 100 µl/flask of diluted bacterial suspension to obtain a MOI of 0.1 to 10 depending on your experimental conditions.

3.3 Culture of *THP1* and infection assays

1. Cultivate THP1 cells in RPMI supplemented medium at 37°C in a humid atmosphere containing 5% CO₂. Growth THP1 in 75 cm² cell culture flasks.
2. Split the culture every week: centrifuge the cell suspension 5 min at 500 g room temperature (RT), remove the medium and adjust the cell concentration at 2x10⁵ cells/ml by counting them on a cell chamber slide. Expand the cells depending of the amount needed for the infection assay (*see Note 10*).

3. The day of infection you need 5×10^7 cells/T75 cm² in RMPI medium without FCS per condition (*see Note 11*). Prepare one additional flask for uninfected cells.
4. Infect by adding 100 µl/flask of diluted bacterial suspension to obtain a MOI of 10 to 50 depending on your experimental conditions.

3.4 Sample preparation for sorting experiments

1. For *A. castellanii* infected cells: remove the medium, wash three times with PBS and resuspend the pellet in 10 ml of PBS.
2. For THP1 infected cells: spin the cell suspension 5 min at 500 g RT, wash three times with PBS and resuspend the pellet in 10 ml of PBS.
3. Spin the cell solution 5 min at 500 g RT and resuspend the pellet in 2 ml of FACS buffer (*see Note 12*). Filter the sample through a 50 µm nylon cell strainer (*see Note 13*) onto a sterile, precoated polypropylene tube and immediately perform flow cytometry measurements or store the samples on ice (*see Note 14*).

3.5 Sorting experiments

Sorting setup and experiments described below are optimized for the use of FACSAria II cell sorter, this section should be adjusted depending on the machine used.

1. Setup of the cytometer (fluorescence parameter, nozzle size, sheath pressure, frequency) according to your working conditions. Use for *A. castellanii* an 85 µm nozzle with sheath pressure of 45 psi and a frequency of 47 Hz. For THP1, use a 70 µm nozzle, 70 psi sheath pressure and 87 Hz.
2. Chose the collection tube holder (two-way 15 ml) and prepare the collecting tubes (*see Note 15*).
3. Start the fluidic startup and set up the stream (check that the stream is correctly collected into the waste drawer).
4. Run the Cytometer Set up and Tracking beads (CST) (*see Note 16*).
5. Start by analyzing the uninfected sample to set up the cytometer parameters (*see Note 17*). Acquire the cell suspension and optimize the scatter signal until the cell population is visible in a FSC/SSC plot (*see Note 18*) (**Fig.2A**). Use the gated untreated cell population to define the negative fluorescence signal.

- 185 6. Analyze the infected samples using the predefined gating on the uninfected cells and
186 define the subcellular populations that must be sorted corresponding to infected (GFP-
187 positive) and uninfected cells (GFP-negative)(*see Note 19*)(**Fig.2B**).
- 188 7. Perform the Drop Delay using Accudrop beads and chose your sort mask (*see Note 20*).
- 189 8. Sort the defined gate at room temperature or at 4°C in function of your biological readout
190 onto pre-coated polypropylene tubes (*see Note 21 and 22*). Remember to set the same
191 temperature for the collection and the injection chamber. The sorting time depends on the
192 concentration of your sample, the strain used and on the time of infection.
- 193 9. To evaluate the sorting efficiency as well as the enrichment of your cell population
194 analyze samples corresponding to the positive (**Fig. 2C**) and negative fractions (**Fig. 2D**).
- 195 10. Export the calculated proportions to statistic software to generate graphs and significant
196 values.
- 197 11. Clean the cell sorter as recommended by the facility you are using (we suggest two
198 washes Contrad 70 (or similar), two washes ethanol 70% and two washes water.
- 199 12. Shut down the fluidics.

200

4. Notes

1. The protocol was set up with a pBCKS- P_{flaA} -*gfp* plasmid, but any plasmid harboring a fluorescent protein can be used as long as the cytofluorimeter is able to detect it.
2. Prepare a 500X stock solution of L-cysteine at 200 g/L and a 1000X stock solution of $\text{Fe}(\text{NO}_3)_3$ at 250 g/L. To dissolve the iron solution, warm up at 56°C.
3. We here used a 488-nm blue laser to excite the GFP protein (LP Mirror 502; BP Filter 530/30). You can change the laser or the filters according to the fluorescent protein you are using.
4. As a general rule, for optimal results when sorting large or fragile cells, use a larger nozzle size and lower pressure. To increase throughput and yield when sorting smaller or less fragile cells, use a smaller nozzle size and higher pressure.
5. Cells may be resuspended in any isotopic salt solution calcium and magnesium free.
6. Never sort into dry tubes. Coat them with serum or culture medium at 37°C.
7. *L. pneumophila* life cycle switches between two forms: replicative avirulent and transmissive bacteria expressing virulent traits, such as flagella (11). When translated to in *in vitro* conditions transmissive phase corresponds to post exponential/stationary growth phase: thus one can choose to directly use a bacterial lawn grown on CYE agar plates (for P_{flaA} -*gfp* expressing bacteria in the stationary phase the green color will be visible by eye) or to inoculate 50 ml fresh AYE media at an OD_{600} of 0.1 from an overnight culture and allow bacteria to grow to an $\text{OD}_{600} > 4.2$.
8. The exact correlation of the OD_{600} and the bacterial concentration depends on the spectrophotometer used and has to be determined experimentally.
9. In order to amplify the eukaryotic cells seed $\approx 2\text{-}4 \times 10^5$ cell/ 25 cm^2 flask to obtain an 80-90% confluent culture in one week.
10. An inoculation of 2×10^5 cells/ml will reach 10^6 cells/ml after one week.
11. Infection assays are performed in RPMI without serum to avoid extracellular growth of *L. pneumophila*. However, for short time points of infection (less than 1 replication cycle ≈ 16 hours) the addition of FCS does not impact the bacterial replication.
12. A cell density of $5\text{-}50 \times 10^7$ cells/ml is optimal.
13. Sample filtration reduces the probability of a blockage in the flow cell nozzle.

14. Incubation for a long time before FACS acquisition might impact the bacterial replication state. In this case a fixation step with PBS-4% PFA is suitable before the filtering step. If one wants to isolate mRNA or other small RNAs from sorted cells one might also fix them in RNAlater solution (commonly used to stabilize and protect RNA degradation), however a reduced fluorescence from GFP should be take into account (12) as well as a different setup of scatter gates due to an increased viscosity of the sample (13).
15. The system offers three different collection tube holders: we recommend to use a two-way holder with a 5 ml polypropylene collection tube as the sample will be highly diluted during the sorting.
16. When you run the CST remember to use the adapted filter.
17. The hydrodynamic forces utilized in the operation of the FACS could affect the properties of the cells. It is thus recommended to use the uninfected sorted sample in order to compare properly the output.
18. The intensity of the light scattered in the forward direction (FSC) denotes cell size, while the 90° side scatter (SSC) is refracted in proportion to the granular content of the cell, thus characterizing the intracellular complexity. The FSC/SSC scatter profile might be used to approximately discriminate between live and dead cells: dead cells often have lower forward scatter and higher side scatter than live cells. By drawing a gate that excludes those events with low FSC and high SSC, you can exclude debris and dead cells from the analysis.
19. Depending on your biological readout you may process both GFP-positive and GFP-negative sorted cell populations. However, keep in mind that, depending on the infection time point, the GFP signal may be undetected by the FACS, which may lead to false negative events in the GFP-negative sample. Always double check the negativity of the GFP-samples by plating a sample on BYE agar plates or use uninfected sorted cells as negative control.
20. To set up the precision mode for sorting you must set up a Sort Mask. The choice of the mask depends on the fact if you want to collect your positive cell population in a purity, single cell or recovery mode. In the described conditions we used a Purity BD standard mode (Yield mask: 32/Purity mask: 32/Phase mask: 0).
21. Optimal threshold rate for standard sorting is $\approx 20,500$ events/sec.

262 22. Creation of droplets and aerosols during cell sorting: protection of all laboratory workers
263 from exposure is critical. Owing to the high fluid pressure produced in high-speed cell
264 sorters large amounts of secondary aerosols of various and undefined droplet sizes can
265 occur during instrument failures, for instance, when a partial clog in the nozzle causes a
266 deflection in the fluid stream. Because of the potential health risk to sorter operators and
267 the environment, aerosol containment of the sorter must be verified in compliance with
268 the biological risk of the sample. This is especially true in the case of *L. pneumophila*.
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Figure legends

Figure 1: Principle of cell sorting

FACS is an abbreviation for Fluorescence-Activated Cell Sorting combining a conventional flow-cytometer, collecting forward-scatter, side-scatter and fluorescent signal, to an electromagnetic tunable transducer incorporated in the flow chamber. **A)** Cells or other particles (Sample) are suspended in a liquid stream that runs through the system in a laminar flow. **B)** The sample becomes strung out in a single file fashion in the direction of the flow where it will pass the interrogation point (Laser). Each individual event interacting with the light is measured by an electronic detection apparatus as light scatter and fluorescence intensity. If a fluorescent label, or fluorochrome, is specifically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular cell component. **C)** After interrogation, the stream is portioned into identically sized droplets that are generated by the application of an acoustic wave that causes the fluid stream to break-off into individual droplets that detach from the main stream a few millimeters downstream from the nozzle (Droplet break-off point). The distance in time between the laser interrogation point and the droplet break-off point corresponds to the drop delay value, that determines how long the system must wait before it applies a charge once a target particle is detected. **D)** When a target particle is detected, a charge is applied to the droplet by two strongly charged deflection plates. Electrostatic attraction and repulsion cause each charged droplet to be deflected to the left or right, depending on the droplet's charge polarity, while uncharged droplets pass into the waste.

Figure 2: Example of *Acanthamoeba castellanii* sorting parameters

Both uninfected *Acanthamoeba castellanii* (A) or *A. castellanii* infected six hours with *L. pneumophila* strain Paris harboring a pBCKS-*P_{flaA}-gfp* plasmid (B-D) were analyzed with a FACSaria II. **(A)** Uninfected cells are gated for SSC/FSC patterns to isolate cell populations (P1). SSC-W/SSC-A and FSC-W/FSC-A plots were used for doublet discrimination (P2/P3). **(B)** *A. castellanii* infected cells gated P1/P2/P3 were analyzed for fluorescence staining (FITC-A) and two populations were identified: GFP-positive (P4: 27.6%) and GFP-negative (P5: 68.8%). **(C)** The GFP-positive (P4) and **(D)** the GFP-negative (P5) sorted fractions were

342 analyzed to estimate the cell population enrichment: the positive fraction was enriched from
343 27.6% to 87.5%.