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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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19 **Abstract**

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

31  
32 **Key Words:** *Legionella pneumophila*, phagocytic cells, fluorescent proteins, single cell sorting.

33

## 34 **1 Introduction**

35 The Gram-negative bacterium *Legionella pneumophila* is able to form an intracellular niche in  
36 infected cells allowing it to replicate and then to spread in the extracellular medium to re-infect  
37 new host cells (1). The formation of a vacuole and the hijacking of cellular response require a  
38 functional Type-4 Secretion System (T4SS) that allows the injection of more than 300 effectors  
39 in the host cell, which are then subverting signal transduction and vesicle trafficking pathways  
40 (2).

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

55  
56 Fluorescence-activated cell sorting (FACS) is a widely used tool in biological research that  
57 utilizes laser-based technology to count, sort and profile a heterogeneous population of cells in a  
58 fluid mixture (**Fig. 1**). This technique allows for a rapid and highly accurate quantitation of  
59 individual cells based on differential fluorescence intensities or biological characteristics, such as  
60 size (by forward-scattered light; FSC) and internal structure (by side-scattered light; SSC). The  
61 sorting function allows high-speed, accurate separation and collection of individual cells based on  
62 user-defined flow cytometry parameters. Beyond the exceptionally accurate and analytical nature,  
63 cell sorting is non-destructive with little to no effect on cell viability or function.

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

70

## 71 **2 Materials**

72 Prepare all solutions using ultrapure water and analytical grade reagents.

### 73 **2.1 Strains and growth media**

- 74 1. We used a *L. pneumophila* strain Paris harboring a pBC-KS plasmid encoding for a GFP  
75 protein under the control of the *flaA* promoter (our unpublished data). In these conditions,  
76 the GFP protein is produced in the post-exponential (virulent) phase (*see Note 1*). *L.*  
77 *pneumophila* grows at 37°C and requires L-cysteine and iron.
- 78 2. CYE (charcoal yeast extract) agar plates: 10 g/L ACES, 10 g/L yeast extract in water;  
79 adjust the pH to 6.9 with KOH; add 15 g/L agar and 2 g/L charcoal powder; autoclave and  
80 let the agar solution cool down to 50°C. Add filter sterilized L-cysteine, iron pyrophosphate  
81 solutions (*see Note 2*) and antibiotics. Store the plates at 4°C.
- 82 3. AYE (ACES yeast extract) medium: 10 g/L ACES, 10 g/L yeast extract; adjust the pH to  
83 6.9 with KOH. Filter sterilize using a 0.2 µm filter cartouche and add filter sterilized L-  
84 cysteine, iron pyrophosphate solutions (*see Note 2*) and antibiotics. Store at 4°C.
- 85 4. Antibiotics: 10 µg/ml chloramphenicol (Cam, stock: 100 mg/ml in ethanol).
- 86 5. Dulbecco's phosphate-buffered saline (D-PBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup>.
- 87 6. Spectrophotometer and plastic cuvettes to measure the OD.

88

### 89 **2.2 Acanthamoeba castellanii**

- 90 1. The *Acanthamoeba castellanii* strain ATCC50739 was cultured at 20°C.
- 91 2. PYG 712 medium: rich medium for *A. castellanii* growth. 2% proteose peptone, 0.1% yeast  
92 extract, 0.1 M glucose, 4 mM MgSO<sub>4</sub>, 0.4 M CaCl<sub>2</sub>, 0.1% sodium citrate dihydrate, 0.05

- 93 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, 2.5 mM NaH<sub>2</sub>PO<sub>3</sub>, 2.5 mM K<sub>2</sub>HPO<sub>3</sub>. Sterilize the  
94 medium using a 0.2 μm filter cartouche and store at 20°C.
- 95 3. Infection buffer: PYG 712 medium without proteose peptone, glucose and yeast extract.  
96 This minimal medium is used for intracellular growth assays.
- 97 4. Culture flasks: 25 cm<sup>2</sup> or 75 cm<sup>2</sup>; filter screw cap; growth-enhanced treated; sterilized by  
98 radiation; free from pyrogens, DNA/RNA and DNase/RNase.
- 99 5. Cell counting chamber slides.
- 100 6. Cell incubator set at 20°C.

101

### 102 **2.3 THP-1 macrophages**

- 103 1. The THP1 cell line ATCC TIB202 is used and grown at 37°C and 5% CO<sub>2</sub> in humidified  
104 air in an incubator.
- 105 2. Supplement the commercially available RPMI 1640 GlutaMAX™ Supplement medium  
106 with 10% heat-inactivated fetal calf serum (FCS).
- 107 3. Infection assays were performed in RPMI 1640 GlutaMAX™ Supplement medium without  
108 FCS.
- 109 4. Culture flasks; 25 cm<sup>2</sup> or 75 cm<sup>2</sup>; filter screw cap; growth-enhanced treated; sterilized by  
110 radiation; free from pyrogens, DNA/RNA and DNase/RNase.
- 111 5. Cell counting chamber slides.
- 112 6. Cell culture incubator
- 113 7. Centrifuge equipped with a swing rotor.

114

### 115 **2.4 Cell Sorter**

- 116 1. The cell sorter used is a FACSAria II (Becton Dickinson) equipped with a 488 nm  
117 excitation source (*see Note 3*) and a 70 or 85 μm nozzle (*see Note 4*).
- 118 2. The FSC/SSC and fluorescence signals are acquired and quantified using the FACS DIVA  
119 software (Becton Dickinson) and FlowJo (Treestar).
- 120 3. The samples are resuspended in FACS buffer (PBS; 10 mM HEPES; 2% FCS; 2 mM  
121 EDTA) (*see Note 5*) and measured in sterile polypropylene tubes pre-coated with serum or  
122 culture medium at 37°C (*see Note 6*).
- 123 4. Infected cells might be fixed in PBS-4% paraformaldehyde solution.

124

### 125 **3 Methods**

#### 126 **3.1 Growth of *L. pneumophila***

- 127 1. Streak out *L. pneumophila* from frozen glycerol stocks onto CYE/Cam plates. A bacterial  
128 lawn will form after 3-4 days of incubation at 37°C.
- 129 2. The day of infection suspend a loop of bacteria grown on CYE/Cam plates in 900 µl PBS,  
130 mix thoroughly, measure the OD<sub>600</sub> and adjust the density of the bacterial suspension to  
131 OD<sub>600</sub> of 2 (*see Note 7*). The multiplicity of infection (MOI) is defined as the number of  
132 added bacteria per individual host cell, calculate the bacterial concentration of your  
133 suspension, given that an OD<sub>600</sub> of 2.2 corresponds to approximately 2x10<sup>9</sup> bacteria/mL  
134 (*see Note 8*).

135

#### 136 **3.2 Culture of *A. castellanii* and infection assays**

- 137 1. Cultivate *A. castellanii* in PYG medium at 20°C in culture flasks (25 cm<sup>2</sup> or 75 cm<sup>2</sup>  
138 depending on the number of cells needed).
- 139 2. Split the culture every week: remove PYG medium, add 10 ml of PBS, tap off the cells,  
140 and count them on a cell counting chamber slide. Expand the cells depending on the  
141 amount needed for the infection assay (*see Note 9*).
- 142 3. The day of infection seed 10<sup>7</sup> cells/T75 cm<sup>2</sup> culture flasks in 10 ml of infection medium  
143 per condition. Let the cells settle and adhere to the plastic surface for 1-2 hours. Prepare  
144 one additional flask for uninfected cells.
- 145 4. Infect by adding 100 µl/flask of diluted bacterial suspension to obtain a MOI of 0.1 to 10  
146 depending on your experimental conditions.

147

#### 148 **3.3 Culture of *THP1* and infection assays**

- 149 1. Cultivate THP1 cells in RPMI supplemented medium at 37°C in a humid atmosphere  
150 containing 5% CO<sub>2</sub>. Growth THP1 in 75 cm<sup>2</sup> cell culture flasks.
- 151 2. Split the culture every week: centrifuge the cell suspension 5 min at 500 g room  
152 temperature (RT), remove the medium and adjust the cell concentration at 2x10<sup>5</sup> cells/ml  
153 by counting them on a cell chamber slide. Expand the cells depending of the amount  
154 needed for the infection assay (*see Note 10*).

- 155 3. The day of infection you need  $5 \times 10^7$  cells/T75 cm<sup>2</sup> in RPMI medium without FCS per  
156 condition (*see Note 11*). Prepare one additional flask for uninfected cells.
- 157 4. Infect by adding 100 µl/flask of diluted bacterial suspension to obtain a MOI of 10 to 50  
158 depending on your experimental conditions.

### 159 **3.4 Sample preparation for sorting experiments**

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

168

### 169 **3.5 Sorting experiments**

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

- 172 1. Setup of the cytometer (fluorescence parameter, nozzle size, sheath pressure, frequency)  
173 according to your working conditions. Use for *A. castellanii* an 85 µm nozzle with sheath  
174 pressure of 45 psi and a frequency of 47 Hz. For THP1, use a 70 µm nozzle, 70 psi sheath  
175 pressure and 87 Hz.
- 176 2. Chose the collection tube holder (two-way 15 ml) and prepare the collecting tubes (*see*  
177 **Note 15**).
- 178 3. Start the fluidic startup and set up the stream (check that the stream is correctly collected  
179 into the waste drawer).
- 180 4. Run the Cytometer Set up and Tracking beads (CST) (*see Note 16*).
- 181 5. Start by analyzing the uninfected sample to set up the cytometer parameters (*see Note 17*).  
182 Acquire the cell suspension and optimize the scatter signal until the cell population is  
183 visible in a FSC/SSC plot (*see Note 18*) (**Fig.2A**). Use the gated untreated cell population  
184 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

## 201 4. Notes

- 202 1. The protocol was set up with a pBCKS- $P_{flaA}$ -*gfp* plasmid, but any plasmid harboring a  
203 fluorescent protein can be used as long as the cytofluorimeter is able to detect it.
- 204 2. Prepare a 500X stock solution of L-cysteine at 200 g/L and a 1000X stock solution of  
205  $\text{Fe}(\text{NO}_3)_3$  at 250 g/L. To dissolve the iron solution, warm up at 56°C.
- 206 3. We here used a 488-nm blue laser to excite the GFP protein (LP Mirror 502; BP Filter  
207 530/30). You can change the laser or the filters according to the fluorescent protein you  
208 are using.
- 209 4. As a general rule, for optimal results when sorting large or fragile cells, use a larger  
210 nozzle size and lower pressure. To increase throughput and yield when sorting smaller or  
211 less fragile cells, use a smaller nozzle size and higher pressure.
- 212 5. Cells may be resuspended in any isotopic salt solution calcium and magnesium free.
- 213 6. Never sort into dry tubes. Coat them with serum or culture medium at 37°C.
- 214 7. *L. pneumophila* life cycle switches between two forms: replicative avirulent and  
215 transmissive bacteria expressing virulent traits, such as flagella (11). When translated to  
216 in *in vitro* conditions transmissive phase corresponds to post exponential/stationary  
217 growth phase: thus one can choose to directly use a bacterial lawn grown on CYE agar  
218 plates (for  $P_{flaA}$ -*gfp* expressing bacteria in the stationary phase the green color will be  
219 visible by eye) or to inoculate 50 ml fresh AYE media at an  $\text{OD}_{600}$  of 0.1 from an  
220 overnight culture and allow bacteria to grow to an  $\text{OD}_{600} > 4.2$ .
- 221 8. The exact correlation of the  $\text{OD}_{600}$  and the bacterial concentration depends on the  
222 spectrophotometer used and has to be determined experimentally.
- 223 9. In order to amplify the eukaryotic cells seed  $\approx 2\text{-}4 \times 10^5$  cell/25  $\text{cm}^2$  flask to obtain an 80-  
224 90% confluent culture in one week.
- 225 10. An inoculation of  $2 \times 10^5$  cells/ml will reach  $10^6$  cells/ml after one week.
- 226 11. Infection assays are performed in RPMI without serum to avoid extracellular growth of  
227 *L. pneumophila*. However, for short time points of infection (less than 1 replication cycle  
228  $\approx 16$  hours) the addition of FCS does not impact the bacterial replication.
- 229 12. A cell density of  $5\text{-}50 \times 10^7$  cells/ml is optimal.
- 230 13. Sample filtration reduces the probability of a blockage in the flow cell nozzle.

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

269

270

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## 312 **Figure legends**

### 313 **Figure 1: Principle of cell sorting**

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

333

### 334 **Figure 2: Example of *Acanthamoeba castellanii* sorting parameters**

335 Both uninfected *Acanthamoeba castellanii* (A) or *A. castellanii* infected six hours with  
336 *L. pneumophila* strain Paris harboring a pBCKS-*P<sub>flaA</sub>-gfp* plasmid (B-D) were analyzed with a  
337 FACSaria II. **(A)** Uninfected cells are gated for SSC/FSC patterns to isolate cell populations  
338 (P1). SSC-W/SSC-A and FSC-W/FSC-A plots were used for doublet discrimination (P2/P3).  
339 **(B)** *A. castellanii* infected cells gated P1/P2/P3 were analyzed for fluorescence staining  
340 (FITC-A) and two populations were identified: GFP-positive (P4: 27.6%) and GFP-negative  
341 (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%.





