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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 Ca²⁺ and Mg²⁺.
- 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 MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05

- 93 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, 2.5 mM NaH₂PO₃, 2.5 mM K₂HPO₃. 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 cm² or 75 cm²; 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% CO₂ 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 cm² or 75 cm²; 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 OD₆₀₀ and adjust the density of the bacterial suspension to
131 OD₆₀₀ 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 OD₆₀₀ of 2.2 corresponds to approximately 2x10⁹ 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 cm² or 75 cm²
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 10⁷ cells/T75 cm² 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% CO₂. Growth THP1 in 75 cm² 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 2x10⁵cells/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 5×10^7 cells/T75 cm² in RMPI 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 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 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 cm^2 flask to obtain an 80-
224 90% confluent culture in one week.
- 225 10. An inoculation of 2×10^5 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 ≈ 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 during 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 \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*.

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_{flaA} -*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%.



