JAN 25, 2023

# 🌐 Protocol for high-throughput isolation of bacterial intracellular nonreplicating persisters

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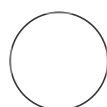
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DISCLAIMER

There are no competing interests relating to employment, consultancy, patents, products in development, or marketed products. We present a full and objective manuscript that has no interference to its objectivity.

Protocol status: Working
We use this protocol and it's working

Created: Jan 12, 2023

Last Modified: Jan 25, 2023

ABSTRACT

Failure of antibiotics to clear a bacterial infection is arguably one of the most severe threat to global health. Bacteria ability to withstand drug treatment partly relies on the formation of a subpopulation of persisters. Persisters are rare nonreplicating individuals, transiently refractory to antibiotic treatment and responsible for non-resolving bacterial infection and relapse. Studying persisters is challenging as it is a reversible physiological state occurring in a small fraction of the pathogen population hidden in a complex environment. Persisters have been primarily analyzed in broth, hardly reflecting the conditions prevailing within a host. In addition, indirect read-outs used to detect the persisters, *a posteriori*, limited a comprehensive understanding of the persisters peculiar physiology during the infection. Here, we describe a high-throughput method to track, and collect the bacterial persisters generated during the infection of phagocytic cells, with single-cell resolution. Persisters are detected based on the bacterial division rate measured for each individual bacterium using the Timer fluorescent growth rate reporter and subsequently recover using fluorescence-activated cell sorting (FACS). We will illustrate this method with *Legionella pneumophila*, a facultative intracellular pathogen and the agent of the Legionnaires' disease for which persistence to antibiotics and relapsing infections are clinically documented. Isolated persisters can be analyzed biochemically and functionally, yielding fundamental new insights into the molecular and cellular processes underlying persistence, and into the peculiar physiology of the persisters within the host. This method is adaptable to other microbial pathogens and alternative infection models.

ATTACHMENTS

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GUIDELINES

SUMMARY:

Persisters represent a subpopulation of nonreplicating individuals transiently refractory to antibiotic treatment. Here we describe a high-throughput single-cell method to track and collect the persisters formed within phagocytes. Cells are infected with bacteria expressing the Timer fluorescent growth rate reporter, and nonreplicating individuals are recovered using fluorescence-activated cell sorting (FACS).

INTRODUCTION:

During the colonization of the host, the pathogen encounters highly heterogeneous host micro-environments [1], elaborate a complex interplay with the component of the immune systems [2] and/or develops microbial communities with high degree of phenotypic specialization [3] which produce, within the micrometer ranges, a

multitude of genetically identical individuals with alternative physiologies [4]. Bacterial variability is further increased as clonal bacteria constantly develop functionally distinct subpopulations [5]. This reversible and regulated phenomenon termed phenotypic heterogeneity is of major clinical importance as it implies the formation of persisters [6]. Persisters are preestablished or stress induced individuals that transiently evade the bactericidal activity of antibiotics by entering a rare and peculiar physiological state at the cost of abrogation of bacterial division, and finally resume growth on the onset of the treatment termination [7]. The emergence of persisters has been documented for major bacterial pathogens including Staphylococcus aureus, Mycobacterium tuberculosis, Escherichia coli, Salmonella enterica, Pseudomonas spp., Listeria monocytogenes and Legionella pneumophila [4,6,8–16]. Traditionally, the presence of bacterial persisters has been revealed by the "biphasic killing" phenomenon of bacterial cultures exposed to bactericidal antibiotics [17]. However, such population-averaging methods, performed a posteriori, are poorly suited to comprehensively and dynamically analyze a phenomenon where reversible cell-to-cell variations plays a key role. The development of high-throughput fluorescence-based single-cell technologies has rendered important breakthroughs reachable. Notably, the microfluidic device called mother machine has been incredibly useful to directly monitor the formation of persisters and the underlying regulatory mechanisms using a combination of genetic and environmental manipulations [6]. Persisters have been mostly studied using non-infectious model organisms cultivated in broth, hardly reflecting the conditions prevailing within a host. Deciphering the biology of the persisters during the infection has remained technically challenging as the persisters represent a rare and unstable subpopulation of the pathogen, that is generated within complex environments.

The ubiquitous environmental Gram-negative bacterium Legionella pneumophila is a waterborne human pathogen responsible for a life-threatening pneumonia termed Legionnaires' disease (LD) [18], the inventoried cases of which have doubled since 2013 [19]. LD is fatal for 5-15% of the patients (up to 50% among the elderly), even when promptly diagnosed and treated [20]. A nation-wide retrospective study describing those cases of slowly or non-resolving LD proposed that recalcitrance to antibiotic treatments or relapsing infections were the expression of bacterial persistence [21]. L. pneumophila survives its ingestion by a diverse array of protozoan predators, as well as lung alveolar macrophages, and establishes a replicative membrane-bound compartment termed the Legionella-containing vacuole (LCV) [22,23]. LCV formation requires the Icm/Dot type IV secretion system (T4SS) and involves approximately 300 different so-called « effector » proteins, which are translocated into host cells [24,25] to hijack numerous host cell functions. The amoebozoa Acanthamoeba castellanii has proved a reliable, easy-to-use and cost effective model organism to investigate the molecular interactions of L. pneumophila with its host cell, and at large, the pathogenic potential and process of many other human opportunistic pathogens [26].

The plasmid based Timer growth rate reporter [4] is a powerful technology to track the nonreplicating persisters produced during an infection. Timer is a stable fluorescent protein that slowly matures from a green to a red fluorescent protein. As a consequence, in growing bacteria, constitutively producing Timer, green fluorescent Timer dominates over red fluorescent Timer, which is diluted by cell division before maturation, and the individual bacteria show a high [500 nm (green)/600 nm (red)] fluorescence (color) ratio (i.e. "green" fluorescence). By contrast, growth-arrested bacteria accumulate both green and red fluorescent Timer, and the individual bacteria show a low green/red color ratio (i.e., "red" fluorescence).

Here, coupling the protozoan model of infection, the innovative Timer fluorescent growth rate reporter and high-throughput single-cell technologies, we are defining a pipeline to track and collect the persisters produced by L. pneumophila with an unprecedented resolution. Isolated persisters can be analyzed biochemically and functionally. To date, using this methodology, we demonstrated that L. pneumophila persisters are a preestablished subpopulation of nongrowers and the expression of a regulated pathogen strategy which is applied to evolutionarily distant host cells (i.e., protists and macrophages) [15,16]. Near in situ biochemical characterization of the intracellular L. pneumophila persisters unveiled a unique physiology and, remarkably, a specific virulence program in order to survive the combined action of the host defenses and the antibiotics, hence, breaking the dogma that persisters are dormant bacteria [15]. Of note, this approach can be applied to the study nonreplicating persisters produced by other pathogens during the infection or within complex microbial communities.

DISCLOSURES:

The authors have nothing to disclose.

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Materials

- Isobiotech ball homogenizer (http://www.isobiotec.com)
- FACSAria III sorter
- BBL yeast extract
- Bacto Proteose Peptone
- D(+)glucose monohydrate
- $MgSO_4$
- $CaCl_2$
- sodium citrate
- $Fe(NH_4)_2(SO_4)_2$
- Na_2HPO_4
- KH_2PO_4
- Bacto™ yeast extract
- agar
- L-cysteine
- $Fe(NO_3)_3$
- activated charcoal powder
- KOH
- *N*-(2-acetamido)-2-aminoethane-sulfonic acid
- glycerol solution
- chloramphenicol

Cultivation of *Acanthamoeba castellanii*: Axenic cultivation...

1

Note

Note 1: A. castellanii *strain Douglas (ATCC 30234) is used.* A. castellanii *is an ubiquitous amoeba with a 2-stage lifecycle: an active trophozoite stage that exhibits vegetative growth and a stress induced dormant cyst stage with minimal metabolic activity* [27]*. Only* A. castellanii *trophozoites are suited for infection.*

Note 2: **Warning.** A. castellanii *is an opportunistic pathogen producing rare and severe human infections. Therefore, it must be manipulated in a biosafety containment level 2 in a microbial safety cabinet in compliance with local rules.*

Prepare Peptone Yeast Glucose (PYG) broth (see **Note 3**):

A	B

	A	B
	BBL yeast extract	1 g/L
	Bacto Proteose Peptone	20 g/L
	D(+)glucose monohydrate (50 mL of a 2 M	1.8% (w/v)
	MgSO4 (10 mL of a 0.4 M solution)	4 mM
	CaCl2 (8 mL of a 0.05 M solution)	0.4 mM
	sodium citrate (3.4 mL of a 1 M solution)	3.4 mM
	Fe(NH4)2(SO4)2 (20 mg)	0.05 mM
	Na2HPO4 (10 mL of a 0.25 M solution)	2.5 mM
	KH2PO4 (10 mL of a 0.25 M solution)	2.5 mM

Add the components (except the glucose) to ⚗ 950 mL H$_2$O, adjust the pH with [M] 1 Molarity (M) HCl to 6.5±0.1 and autoclave. Solubilize ⚗ 11 g of D(+)glucose in ⚗ 50 mL of warmed H$_2$O (🌡 37 °C) and add to the autoclaved medium (see **Note 4**). Sterilize the medium using a ⊣⊢ 0.2 µm filter cartouche and store at 🌡 4 °C .

> **Note**
>
> Note 3: _For a high reproducibility of growth characteristics, the components should be tested beforehand, and the same suppliers and batches should be used for all experiments._
> Note 4: _Glucose caramelizes upon autoclaving in combination with the medium. Suspend the D(+)glucose in warmed_ ⚗ 50 mL _of H$_2$O, filter sterilize (0.2 µm) and add to the autoclaved medium._

2 Thaw an ampule of frozen amoeba at 🌡 Room temperature and aseptically transfer the contents into a 13 mL sterile Falcon round-bottom polypropylene tubes containing ⚗ 10 mL of PYG. Centrifuge ⊛ 250 x g, 00:10:00 and discard the supernatant. Resuspend the pellet in ⚗ 10 mL of PYG medium to inoculate a tissue culture treated flask (75 cm^2: 10-15mL medium) for initial amplification.

10m

3 Cultivate the amoebae at 🌡 23 °C in PYG medium optionally supplemented with antibiotics (e.g. Pen/Strep or Fungizone) to avoid bacterial and fungal contaminations. Use either 100 mm tissue culture treated Petri dishes (minimum of ⚗ 10 mL medium) or tissue culture treated flasks (25 cm^2: 5-7 mL medium, 75 cm^2: 10-15mL medium). Change the medium every 2-3 days. Split the culture when cell reach 80% confluence by repeated pipetting of the media over the

plate.

Cultivation of *Acanthamoeba castellanii*: Cryopreservation ...

4 Remove the PYG medium and harvest the amoeba, when in logarithmic growth phase, by repeated pipetting of fresh refrigerated PYG medium over the plate. Determine the cell concentration by counting the amoeba with a haemocytometer. Adjust the concentration to about 10^7 amoeba/mL.

5 Prepare freshly a 15% solution of sterile dimethyl sulfoxide (DMSO) in refrigerated PYG medium and mix to the cell suspension in equal portions to reach a final concentration between 10^6 and 10^7 cells/mL and 7.5 % DMSO.

6 Dispense in ⚗ 0.5 mL aliquots into 2.0 mL sterile plastic screw-capped CryoTube vials. Place the vials in a Nalgene Cryo 🌡 1 °C freezing container and store at 🌡 -80 °C , 🕐 Overnight .

> **Note**
>
> For long-term storage, the cell preparation shall be deposited in either the liquid or the vapor phase of a nitrogen freezer.

Generation of a Timer producing *Legionella pneumophila*

7

> **Note**
>
> *Note 5:* **Warning.** L. pneumophila *JR32 is used. This lab strains originally derived from a Philadelphia-1 clinical strain isolated from an outbreak in Philadelphia in 1976* [28,29]*.* L. pneumophila *is the causing agent of the Legionnaires' disease and must therefore be manipulated in a biosafety containment level 2 in a microbial safety cabinet in compliance with local rules.*
> *Note 6:* L. pneumophila *Δ*icmT *lacks a functional T4SS and therefore cannot grow within infected cells. It is a convenient control to define the Timer spectral properties corresponding to the lack of bacterial division during the infection.*

Prepare CYE (charcoal yeast extract) agar plates:

A	B
ACES	10 g/L
Bacto™ yeast extract (see Note 7)	10 g/L
activated charcoal powder (puriss. p.a.)	2 g/L
agar	15 g/L
L-cysteine	3.3 mM
Fe(NO3)3	0.6 mM

Dissolve ⚗ 10 g of ACES and ⚗ 10 g of yeast extract in ⚗ 950 mL of H_2O and adjust the pH to ⦿ 6.9 with [M] 10 Molarity (M) KOH. Transfer the solution to a 1 L Schott bottle containing ⚗ 2 g of activated charcoal powder, ⚗ 15 g of agar and a stir bar. Autoclave and let the agar solution cool down to 🌡 50 °C . Add filter sterilized ⚗ 0.4 g / ⚗ 10 mL L-cysteine and ⚗ 0.25 g / ⚗ 10 mL Fe(NO$_3$)$_3$ solutions (*see* **Note 8**). If required add antibiotics. Mix the solution on a magnetic stirrer and pour plates (approximately 40 plates per L of medium). Let the plates dry for 1 day at 🌡 Room temperature and store at 🌡 4 °C .

> **Note**
>
> *Note 7:* The source and quality of yeast extract and peptone affect the physiology of *L. pneumophila*. *For a high reproducibility of virulence traits and growth characteristics, the components should be tested beforehand, and the same suppliers and batches should be used for all experiments.*
>
> *Note 8:* Dissolve L-cysteine and Fe(NO$_3$)$_3$ each separately in 10 mL of H_2O in a 15 mL tube.
>
> *Stir the medium and slowly add the L-cysteine solution first, followed by the iron solution to prevent precipitation.*

8   **Prepare ACES yeast extract (AYE) broth**:

A	B
N-(2-acetamido)-2-aminoethane-sulfonic acid (ACES)	10 g/L
Bacto yeast extract	10 g/L
L-cysteine	3.3 mM
Fe(NO3)3	0.6 mM

Add ⟁ 10 g of ACES and ⟁ 10 g of yeast extract in ⟁ 950 mL of H_2O. Add filter sterilized ⟁ 0.4 g / ⟁ 10 mL L-cysteine and ⟁ 0.25 g / ⟁ 10 mL $Fe(NO_3)_3$ solutions. Adjust the pH to ⊙ 6.9 with [M] 10 Molarity (M) KOH. If required, add antibiotics. Pass the medium several times through a glass-fibers filters, and finally sterilize the medium using a →|← 0.2 μm filter cartouche. Store the medium at ❄ 4 °C in the dark (*see* **Note 9**).

> **Note**
>
> *Note 9:* Pre-filter the medium 6 to 8 times through a glass microfibers *filter paper to remove precipitates. L-cysteine is light sensitive.*

Generation of a Timer producing *Legionella pneumophila*: P..

9

> **Note**
>
> *Note 10: Perform all steps at room temperature unless stated otherwise.*

Streak out *L. pneumophila* wild-type and the isogenic mutant D*icmT* from frozen glycerol stocks onto CYE plates. Colonies or a bacterial lawn will form after 3 days of incubation at ❄ 30 °C (*see* **Note 11**).

> **Note**
>
> *Note 11: Electrocompetence of* L. pneumophila *is increased when using freshly prepare bacteria grown at 30°C.*

10 Generously harvest the bacterial lawn with a disposable inoculation loop and inoculate ⟁ 1 mL of PBS in a 1.5 mL sterile Eppendorf tube.

11 Centrifuge at ⊛ 5000 rpm, 00:10:00 , discard the supernatant and resuspend the bacterial pellet `10m` with a 10 % glycerol solution. Repeat the operation 3-4 times.

12 Finally, resuspend the bacterial pellet with [⏳ 100 mL] of a 10% glycerol solution.

13 Directly proceed with the electroporation.

Generation of a Timer producing *Legionella pneumophila*: E..

14 Add [⏳ 100 ng] of the plasmid pNP107 to the bacterial suspension and transfer into a 2 mm electroporation cuvette.

15 Electroporate using the following settings: U=2.5 kV; C= 25 µF; 200 Ω=∞; t.c.≈2.5.

16 Immediately add [⏳ 500 µL] of pre-warmed AYE, transfer into a 13 mL Falcon round-bottom polypropylene tubes and incubate on a shaker set at [↻ 200 rpm, 37°C, 05:00:00] .

17 Plate on CYE agar supplemented with [⏳ 5 µg/mL] chloramphenicol (*see* **Note 12**). Orange colonies (*i.e.,* producing the Timer fluorescent protein) will form after 3 days of incubation at [🌡 37 °C] .

> **Note**
>
> *Note 12: Chloramphenicol stock:* [M] 30 mg/mL ethanol. *Store the stock solutions at* [🌡 -20 °C] .

Preparation of infectious Legionella pneumophila

18 Streak out Timer producing *L. pneumophila* from frozen glycerol stocks onto CYE plates supplemented with △ 5 µg/mL chloramphenicol. Colonies or a bacterial lawn will form after 2-3 days of incubation at 🌡 37 °C .

19 Prepare planktonic cultures for infection (*see* **Note 13**). Suspend a loop of the bacterial lawn grown on CYE/Cam agar plates for 2-3 days in △ 600 µL of AYE medium supplemented with △ 5 µg/mL chloramphenicol (AYE/Cam) in a 2 mL Eppendorf tube. Mix thoroughly until you reach a homogeneous suspension (*see* **Note 14**). Measure the $OD_{600\ nm}$ and adjust the density of the bacterial suspension to an $OD_{600\ nm}$ of 0.1. Inoculate △ 9 mL of AYE/Cam with the suspension to obtain an $OD_{600\ nm}$ of 0.1 and split into 3 x △ 3 mL in 13 mL Falcon^{TM} round-bottom polypropylene tubes and incubate on a shaker set at ↻ 200 rpm, 37°C, 21:00:00 .

> **Note**
>
> *Note 13:* L. pneumophila *is most virulent (so-called transmissive) at the transition from the exponential to the stationary growth phase. The appearance of brown pigmentation is an indicator for stationary growth phase.*
> *Note 14: Day 3 bacterial lawn is difficult to dissolve in broth. To prevent the formation of aggregates, first collect the bacterial lawn in a 2 mL Eppendorf tube. Second, add the culture medium and wait for a few minutes prior to mixing.*

20 At 21 h, *L. pneumophila* must be transmissive. Check the bacteria by examining △ 10 µL of culture loaded on a glass slide covered with a 12 mm round glass coverslip with an inverted light microscope (40 × objective). *L. pneumophila* look like a dense population of small and motile coccobacilli (*see* **Note 15**). Measure the $OD_{600\ nm}$; an $OD_{600\ nm}$ of 5 corresponds to approximately 2×10^9 bacteria/mL (*see* **Note 16**).

> **Note**
>
> *Note 15: Presence of elongated bacilli or filaments signals sub-optimal culture quality.*
> *Note 16: Under our conditions an $OD_{600\ nm}$ of 5.0 corresponds to approximately 2×10^9 bacteria/mL. At 21 h, the exact correlation of the $OD_{600\ nm}$ with the bacterial concentration depends on the spectrophotometer used and must be determined experimentally.*

21 Proceed with infection of *A. castellanii*.

22 Split *A. castellanii* cultures one day before the experiment to obtain cells in logarithmic growth phase, which have been fed the day before (*see* **Note 17**).

> **Note**
>
> *Note 17: For best and most reproducible results use* A. castellanii *growing exponentially. Non-dividing, dense or starved cells will be infected less efficiently and less reproducibly.*

23 Wash exponentially growing *A. castellanii* with PYG and detach the cells by repeated pipetting of the media over the plate.

24 Determine cell concentration by counting the cells with a haemocytometer.

25 Seed cells at a density of 9×10^5 amoeba per well of a tissue culture treated 6-well plate. Let the phagocytes settle and adhere to the plastic surface for ⏱ 24:00:00 under normal culture conditions (*see* **Note 18**).

1d

> **Note**
>
> *Note 18: Use* ⚗ 2 mL *of PYG medium per well. The next day, cell number will have doubled. Alternatively, cell can be seeded the day of the infection as cell adhesion to the plastic surface takes* ⏱ 00:30:00 *.*

26 Prior to infection, remove the PYG medium and wash out antibiotics if necessary (*see* **Note 19**). Determine the cell numbers per well using a haemocytometer. Calculate the appropriate number of bacteria to infect host cells with a multiplicity of infection (MOI) of 2.

27 Synchronize the infection by centrifugation of the 6-well plate for ⊛ 250 x g, 00:10:00 and `1d 0h 10m`

incubate for 45 to 60 min at 🌡 25 °C . Remove not internalized bacteria by washing.
- Wash the infected cells with PYG. (1/3)
- Wash the infected cells with PYG. (1/3)
- Wash the infected cells with PYG. (1/3)
- Incubate at least ⏱ 24:00:00 at 🌡 25 °C (*see* **Note 20 & 21**).

Note

Note 20: Subpopulations of proliferative L. pneumophila *and nonreplicating persisters of are distinctly observed and were characterized at 24 h post-infection (p.i.), in infected* A. castellanii*.*
Note 21: Infected A. castellanii loses motility and develops tomette-like shape.

28 Proceed with the sample preparation for FACS.

Sample preparation for FACS

29

Note

Note 22: After host cell lysis, maintain the samples at 4°C to slow down the Timer maturation. Thereby the Timer fluorescent ratio will accurately correlate to the bacterial growth rate for extended period of times (several hours).
Note 23: Optimal lysis of infected A. castellanii *requires a 2-step process combining detergent and mechanical forces.*

Prepare homogenization (HS) buffer:

A	B
N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)	20 mM

A	B
Sucrose	250 mM
Ethyleneglycoltetraacetic acid (EGTA)	0.5 mM
pH adjusted to 7.2 with KOH	1 M

Filter sterilize and store at 🌡 4 °C .

30 🌡 On ice , detach the infected amoeba by repeated pipetting of the media over the wells and transfer into 2 mL Eppendorf tubes.

31 Pellet by centrifugation ⊛ 250 x g, 4°C, 00:10:00 , remove the supernatant and wash once with ⚗ 1 mL ice-cold PBS. **10m**

32 🌡 On ice , lyse the cell pellet with ⚗ 1 mL 0.1 % Triton TX-100 (Sigma) in HS buffer for ⏱ 00:10:00 . **10m**

33 Centrifuge the cell lysate ⊛ 250 x g, 4°C, 00:15:00 , remove the supernatant and resuspend the pellet with ⚗ 1 mL PBS. Keep 🌡 On ice . **15m**

34 Assemble and place the Isobiotech ball homogenizer (http://www.isobiotec.com) (6 or 8 mm clearance ball) 🌡 On ice . Mount a pair of Luer-lock syringes.

35 Rinse well the ball homogenizer.

35.1 Remove the plunger of one syringe and fill the volume with ddH$_2$O. Install the plunger and flush. (1/3)

35.2 Remove the plunger of one syringe and fill the volume with ddH$_2$O. Install the plunger and flush. (2/3)

35.3 Remove the plunger of one syringe and fill the volume with ddH$_2$O. Install the plunger and flush. (3/3)

35.4 Finally flush the homogenizer once with ice-cold HS buffer. Make sure there is no leakage or clogging.

36 Install a new pair of Luer-Lock syringes. Remove the plunger of one syringe to load the cell lysate. Install the plunger and press the sample through the homogenizer into the second syringe. Press back and forth nine times (*see* **Note 24**).

> **Note**
>
> Note 24: **Warning.** *Homogenization generates aerosol that poses a threat to the operator when working with respiratory pathogens. Wear mask and goggles. Operate in biosafety containment level 2 in a microbial safety cabinet. Notify your colleagues.*

37 Collect the homogenized sample and transfer trough the cell-strainer cap into a 5 mL polystyrene round-bottom tube (*see* **Note 25**). Keep 🌡 On ice .

> **Note**
>
> Note 25: *The cell-strainer cap retains oversize particles and therefore protect the cytometer microfluidics from clogging.*

38 Proceed with the isolation of the nonreplicating persisters.

39

Start up the sorter.

39.1 Decontaminate the sort block, the sort chamber, the sample injection port and the sort collection device (tube holder and lid) with 70% ethanol. Clean the deflecting plates with ddH$_2$O. Wipe to dry carefully all surfaces that will be charged.

39.2 Assemble and filled the sheath tank with sterile PBS (_see_ **Note 28 & 29**).

> **Note**
>
> _Note 28:_ Use autoclaved tank.
> _Note 29:_ The sheath level sensor must be decontaminated with 70 % ethanol.

39.3 Turn on the aerosol management system.

39.4 Turn on the sorter (_see_ **Note 30**).

> **Note**
>
> _Note 30:_ Ensure the excitation laser 488 nm and 561 nm are set to "ON".

39.5 In the FACSDiva menu, turn on the fluidic system by selecting "Fluidic startup"(*see* **Note 31**) and follow the instructions.

> **Note**
>
> *Note 31:* Ensure that the lid of the sheath tank is properly closed and the tank pressurized.

39.6 In the sort block, remove the close-loop nozzle and insert the nozzle with an orifice of 70 mm at the dedicated position at the lower end of the cuvette flow cell. Set the correct configuration for the 70 mm nozzle in the FACSDiva menu "view configurations".

39.7 Turn on the external temperature control system for the collection tube holder and set the temperature to 🌡 4 °C . In the FACSDiva menu "Cytometer", set the agitation of the sample in the injection chamber to ⟳ 300 rpm and temperature to 🌡 4 °C .

40 Establish a stable stream and set up the drop formation.

> **Note**
>
> *Note 32:* During sorting, drop drive energy is applied to the stream to break it into highly uniform droplets containing particle of interest. Droplets detach from the stream a few millimeters downstream from the nozzle
> *Note 33:* In the FACSDiva software, use the breakoff window to control the stream and the drop formation.

40.1 Start the stream that should appear in the breakoff window.

40.2 Within the sort block, ensure the stream hit the center of the waste aspirator (*see* **Note 34**).

> **Note**
>
> *Note 34:* The entire sort block assembly can be rotated on a fixed pivot point to adjust the position of the stream in the waste aspirator.

40.3 Stabilize the position of the drop breakoff point by adjusting the amplitude (*see* **Note 35**). Drops before the breakoff point will appear pear-shaped. Ensure satellite drop merge with leading drop.

> **Note**
>
> *Note 35: Each nozzle size configuration is optimized to a preset sheath pressure and also change the related values for drop drive frequency, drop charge levels, laser delay and area scaling. Keep the preset values unless you cannot establish a stable stream (i.e. pressure:* ⊘ 70 undetermined *; gap: 6 and frequency: 87 KHz).*

40.4 Copy the generated Drop 1 value (i.e., the distance between the top of the image and the center of the first broken-off drop) in the target field.

40.5 Turn on the Sweet Spot to maintain the drop breakoff point during the sorting.

40.6 Wait 15-20 min and control the stability of the stream and drop formation. For the Drop 1, a difference of 10 units is acceptable. For the GAP a difference of 1 unit is acceptable.

41 Adjust the drop delay.

> **Note**
>
> *Note 36: The drop delay calibrates the time between the particle analysis and the droplet formation in order to charge the drop the sort.*
> *Note 37: Side streams, deflection, and drop delay are defined using the side stream window in the FACSDiva software.*
> *Note 38: Sorting is controlled using the sort layout window in the FACSDiva software.*

41.1 Ensure the maximal illumination of the central stream using the micrometer dial.

41.2   Turn on the deflection plates (voltage) in the side stream window (*see* **Note 39**).

> **Note**
>
> *Note 39: Warning. High voltage potential. Do not touch the deflection plates when the voltage is ON (red warning light). Keep the sort block door closed.*

41.3   In the FACSDiva menu, create an AccuDrop experiment.

41.4   Place a tube filled with a dilute suspension of AccuDrop beads on the sample injection port and load into the sample injection chamber (*see* **Note 40**). Adjust the flow rate to reach a threshold rate of 2'000-3'000 events per second (*see* **Note 41**).

> **Note**
>
> *Note 40: Tube loading, data acquisition and recording as well as flow rate are controlled using the FACSDiva acquisition dash board.*
> *Note 41: Avoid applying high flow rate.*

41.5   Activate the test sort in the side stream window.

41.6   Activate the optical Filter in the side stream window.

41.7   Adjust the voltage slider to place the left side stream in the center of the box on the left. Ensure that the central stream is visible in the right box (*see* **Note 42**).

> **Note**
>
> *Note 42: Adjust the 2nd, 3rd, and 4th Drop settings to tighten the center stream and fine-tune the side streams, if needed.*

41.8 Stop the test sort in the side stream window.

41.9 In the FACSDiva sort layout window select "2 tube" as device and "Initial" as a precision mode.

41.10 Start sorting the beads by selecting "sort" in the sort layout window (*see* **Note 43**).

> **Note**
>
> *Note 43: Keep the aspirator drawer closed.*

41.11 In the side stream window, adjust the drop delay value in 1-drop increments to achieve close to 100% intensity in the left side stream (*see* **Note 44**).

> **Note**
>
> *Note 44: Alternatively, the drop delay can be set automatically using the Auto Delay button in the side stream window.*

41.12 In the sort layout window, change the precision mode to "Fine Tune" and continue optimizing the drop delay by small increments until the left side stream intensity is > 95 % (*see* **Note 45**).

> **Note**
>
> *Note 45: Wait a few seconds after each small increment for a complete response to the delay change.*

41.13 In the side stream window, inactivate the Optical Filter and set the voltage slider to "0". In the sort layout, stop the sort. Close the AccuDrop experiment.

41.14 Unload the tube filled with AccuDrop beads using the FACSDiva acquisition dash board. Discard the tube or keep it in a fridge for future sort.

42   Set up the side streams for the 2-way sort.

42.1   Insert one 15 mL tube in the 2-way sort tube holder. Close with the universal top. Insert the sort collection device into the sort collection chamber and connect to the temperature control system in order to refrigerate the collection tube.

42.2   In the side stream window, turn on the deflection plates (voltage) and activate "test sort". Optimize the position of the first either right or left side stream using the corresponding voltage sliders (*see* **Note 46**).

> **Note**
>
> *Note 46: Keep the aspirator drawer closed.*

42.3   In the side stream window, open the aspirator drawer to confirm the side stream are properly aligned with the collection 15 mL tube.

42.4   Close the aspirator drawer, inactivate "test sort", turn off the voltage, remove the sort collection device and trash the 15 mL collection tube.

43   Create the gates to sort nonreplicating persisters.

> **Note**
>
> *Note 47: To ensure that your cytometer is performing consistently over time, you should regularly run performance check.*
> *Note 48: Acquisition setup and gating strategy are carried out on the FACSDiva software workplace.*

43.1   In FACSDiva browser, open a new experiment and click on "Cytometer settings".

43.2   In the Cytometer window, in the parameter tab, delete all unneeded optical configuration. Keep forward scatter (FSC); side scatter(SSC); [Laser 488 nm - 502LP - BP 530/30] (FITC) and [Laser 561 nm - 600LP - BP 610/20] (mCherry).

43.3   For each parameter, only record the height (H) of the pulse signal detected.

43.4   In the cytometer window, in the threshold tab, set both the FSC and SSC values to the minimum (i.e., 200).

43.5   On the global worksheet, create (i) a SSC-H vs FSC-H plot; (ii) a FITC-H vs FSC-H plot and (iii) a mCherry-H vs FITC-H plot.

43.6   In the inspector window, select the checkbox for X Axis and Y Axis under biexponential display.

43.7   Place the sample tube filled with homogenized lysates on the sample injection port and load into the sample injection chamber using the FACSDiva acquisition dash board and start acquisition (*see* **Note 49**). Detected signals appear in the plots created on the global worksheet.

> Note
>
> *Note 49:* In the FACSDiva browser / experiment / specimen, make sure that the tube is activated.

43.8   In the cytometer window, parameter tab, set the photomultiplier (PMT) voltage for FSC and SSC in order to center the detected signal on the SSC-H vs FSC-H plot.

43.9 In the cytometer window, parameter tab, set the PMT voltage for FITC-H in order to separate the green Timer signal from the host debris autofluorescence and the electronic noise on the FITC-H vs FSC-H plot (*see* **Note 50**). Using the polygonal gating tools, draw the **Gate 1** around the FITC-H positive population.

> **Note**
>
> *Note 50: The use of homogenized lysate from cell infected with non-fluorescent L. pneumophila confirm the absence of any background particles with similar fluorescence.*

43.10 In the mCherry-H vs FITC-H plot, apply Gate 1. In the cytometer window, parameter tab, set the PMT voltage for mCherry-H in order to center the red Timer signal (*see* **Note 51**).

> **Note**
>
> *Note 51: The use of homogenized lysate from cell infected with mcherry or GFP producing L. pneumophila shall confirm the absence of fluorescence overlap in the mCherry-H vs FITC-H plot. When using a sorter without spatially separated laserlines and detector arrays possible spectral overlap must be corrected via a process of fluorescence compensation.*

43.11 In the mCherry-H vs FITC-H plot, two well-separated subpopulations with similar FITC-H signal intensity appear. The population with the higher mCherry intensity corresponds to the nonreplicating persisters. Using the gating tools, draw the **Gate 2** around this subpopulation (*see* **Note 52 and 53**).

> **Note**
>
> *Note 52: L. pneumophila DicmT cannot replicate intracellularly. To control the gating strategy, use homogenized lysate from cell infected with Timer producing L. pneumophila DicmT mutant. All bacteria shall be contained in **Gate 2**.*
> *Note 53: On the global worksheet verify the gate are properly hierarchized by showing the "population hierarchy view".*

43.12 In the FACSDiva acquisition dash board, select Gate 2 as stopping gate and 10'000 as events to record. Start recording by clicking "record" (*see* **Note 54**).

43.13 In the mCherry-H vs FITC-H plot, using the population hierarchy view, determine the population size in Gate 2 (% Parent).

44 Sort the nonreplicating persisters.

44.1 Insert one 15 mL tube prefilled with ⚗ 3 mL ice-cold PBS in the 2-way sort tube holder. In the sort layout window, define the sort location field to align the collection tube with the side stream as setup in 5.3. In the selected sort location field, add the subpopulation to be sorted (i.e., **Gate 2**). Close with the universal top. Insert the sort collection device into the sort collection chamber (as in 42.1.).

44.2 In the FACSDiva sort layout window select "2 tube" as device and "purity" as a precision mode (*see* **Note 56**).

44.3 In the side stream window, turn on the deflection plates (voltage), open the aspirator drawer and initiate the sample flow ("acquire").

44.4 In the FACSDiva sort layout window, lunch the sort by clicking "sort".

44.5 In the sort layout window, monitor the sorting by observing the sorting rate, the conflicts rate and the sorting efficiency (ideally above 95 %). Inspect regularly the central and side stream in the side stream window.

44.6 Sort until the required number of nonreplicating persisters has been sorted (*see* **Note 57-58**).

> **Note**
>
> *Note 57*: For continuous sorting, select "Continuous" from the Target Events menu in the sort layout window. If sample reloading is requested, pause the sorting, by clicking the "Pause" button in the Sort Layout. The counting of sorted cells is retained when you restart sorting by clicking the "Resume" button.
>
> *Note 58*: Open the sort chamber to visually inspect the 15 mL collection tube filling to avoid spill over. When necessary pause the sort and replace the 15 mL collection tube. Write on the tube the number of nonreplicating persisters sorted and store on ice.

44.7 Stop the sort by inactivating "sort" in the sort layout window and save the "sort report". In the side stream window, control the deflection plates (voltage) are turned off and the aspirator drawer closed.

44.8 Unload the sample tube using the FACSDiva acquisition dash board and eliminate into the dedicated biological wastes disposal in compliance with local rules.

44.9 Open the sort chamber, remove and disassemble the sort collection device to collect and the store 🌡 On ice the 15 mL collection tube.

45 Re-analysis of the sorted nonreplicating persisters.

45.1 In the FACSDiva browser, under experiment and specimen create a new tube (*see* **Note 59**).

45.2 Harvest only 🧪 **100 mL** of sorted nonreplicating persisters from the 15 mL collection tube and transfer into a sample tube.

45.3 Place the sample tube on the sample injection port and load into the sample injection chamber using the FACSDiva acquisition dash board and activate acquisition. Detected signals appear in the plots created on the global worksheet.

45.4 In the FACSDiva acquisition dash board, adjust the flow rate to reach a threshold rate of 2'000-3'000 events per second.

45.5 In the FACSDiva acquisition dash board, select Gate 2 as stopping. Start recording by clicking "record". Stop recording and acquisition after 500-1000 events are recorded (*see* **Note 60**).

45.6 In the mCherry-H vs FITC-H plot, using the population hierarchy view, determinethe population size in Gate 2 (% Parent) to infer the sort purity.

45.7 Unload the sample tube using the FACSDiva acquisition dash board and eliminate into the dedicated biological wastes disposal in compliance with local rules.

45.8 Save your date and proceed with the shut-down.

46   Shut-down.

46.1   Clean by sequentially loading and running sample tubes filled with FACSClean FACSRinse, 70% ethanol and finally sterile ddH$_2$O.

46.2   In the side stream window, turn off the stream, depressurized and disassemble the PBS tank (*see* **Note 61**).

> **Note**
>
> *Note 61: Autoclaved the tank, decontaminate the sheath level sensor with 70 % ethanol.*

46.3   Decontaminate the sort block, the sort chamber, the sample injection port, the sort collection device (tube holder and lid) with 70% ethanol. Clean the deflecting plates with ethanol and ddH$_2$O. Wipe to dry carefully all surfaces.

46.4   Assemble and filled the ethanol tank with 70% ethanol and connect to the sorter fluidics system (*see* **Note 62**).

> **Note**
>
> *Note 62: Ensure that the lid of the ethanol tank is properly closed and the tank pressurized.*

46.5   In the FACSDiva menu, turn off the fluidic system by selecting "Fluidic shutdown" and follow the instructions.

46.6   In the sort block, remove the 70 mm nozzle. Decontaminate the nozzle and its slot at the lower end of the cuvette flow cell with 70 % ethanol. Insert the close-loop nozzle.

46.7   Turn off the external temperature control system and the aerosol management system.

46.8   Turn off the sorter.