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# Mitochondrial Dynamics and Activity in *Legionella*-Infected Cells

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## **Abstract**

The study of *Legionella pneumophila* interactions with host mitochondria during infection has been historically limited by the techniques available to analyze and quantify mitochondrial dynamics and activity in living cells. Recently, new, powerful techniques such as high-content microscopy or mitochondrial respiration assays (*Seahorse*) have been developed to quantitatively analyze mitochondrial parameters. Here we present state-of-the-art methods adapted to analyze mitochondrial dynamics and activity during *Legionella* infection of living human primary macrophages.

## **Key words**

Mitochondrial dynamics, *Legionella pneumophila*, high-content microscopy, Seahorse, mitochondrial respiration assay, mitochondrial fission, mitochondrial morphology, Mitotracker dyes, bioenergetics, living-cell assays

## **1. Introduction**

Mitochondria are eukaryotic organelles in charge of cellular bioenergetics and other essential functions, such as programmed cell death or immune signaling [1]. Their cellular functions make these organelles attractive targets of bacterial pathogens that replicate intracellularly [2], as subversion of metabolism, cell death and immune signaling are instrumental for bacteria to successfully replicate within human cells [3-5]. Mitochondrial functions are strongly influenced by the highly dynamic performance of these organelles, which include dynamic features such as organelle localization, morphology, quantity and activity [6-8].

Mitochondria are highly dynamic organelles that move within eukaryotic cells and change their morphology in order to adapt to energetic requirements of cells, performing fusion events between single organelles to form highly energized elongated mitochondrial networks and fission events that fragment the networks into single organelles with a decreased activity (Fig. 1) [9]. To study mitochondrial dynamics and activity of human cells in health and disease, state-of-the-art techniques widely used in recent years are high-content fluorescent microscopy and mitochondrial respiration assays [3, 10-13]. These two approaches are carried out in living cells and quantitatively analyze mitochondrial morphology and respiration in 384 or 96 well microplates, respectively, which make the results very powerful in terms of conditions analyzed, replicates and statistics, and suitable to be used in high-throughput screening.

Although *Legionella pneumophila* interactions with host mitochondria was first described more than forty years ago [14], limitation of the techniques to study mitochondrial dynamics and activity have delayed the study of the dynamics of *Legionella*-mitochondria interaction until recently [3]. Here we present state-of-the-art methods to analyze mitochondrial dynamics and activity during *Legionella* infection of living human primary macrophages.

## **2. Materials**

### **2.1 Growth of *L. pneumophila* strains**

1. *L. pneumophila* strains constitutively expressing green fluorescent protein (GFP) (*see Note 1*).
2. Kanamycin: resuspend 500 mg in 10 mL of MilliQ water. Make aliquots (50 mg/mL). Store at -20°C. Add to medium at a final concentration of 12.5 µg/mL.
3. Sterile Petri-dishes.
4. N-(2-acetamido)-2-amino-ethanesulfonic acid (ACES)-buffered charcoal-yeast (BCYE) extract agar plate: weigh 37.5 g of N-(2-acetamido)-2-amino-ethanesulfonic acid (ACES)-buffered charcoal-yeast (BCYE) extract agar (pH 6.9, KOH) and transfer to a 1500 mL glass bottle. Add MilliQ sterile water to a volume of 1000 mL and autoclave for 25 min at 121 °C. Cool the agar medium to 55°C in a water bath and complete with 50 mg/mL kanamycin, or as required. Transfer 20 mL agar medium to a 100 mm sterile Petri dish under the conditions of biosafety cabinet. Leave plate at room temperature roughly 30 min to solidify. Store the agar plates in a plastic bag at 4°C until use.
5. 37°C incubator.
6. 10 µL disposable inoculation loops.

### **2.2 Isolation and culture of human monocyte-derived macrophages (hMDMs)**

1. Fetal bovine serum (FBS): heat-inactivate at 56°C for 30 min. Store at -20°C in 50 mL aliquots.

2. Dimethylsulfoxide (DMSO): store at room temperature in the dark.
3. RPMI1640 + Glutamax
4. PBS
5. BSA (low endotoxin)
6. EDTA
7. PBS containing 1% FBS: add 5 mL of FBS in a 500 mL PBS bottle. Store at 4°C for up to one month.
8. PBS containing 0.1% BSA (low endotoxin): add 0.5 mg of BSA in a 500 mL PBS bottle. Store at 4°C for up to one month.
9. Ficoll-Paque solution. Store at room temperature in the dark.
10. Anti-human CD14 antibodies coupled to magnetic beads (Miltenyi Biotec, cat# 130-050-201). Store at 4°C.
11. LS columns (Miltenyi Biotec).
12. QuadroMACS Separator (Miltenyi Biotec)
13. MACS MultiStand (Miltenyi Biotec)
14. Nunc UpCell Surface cell culture 6 well multidish (Sigma).
15. Recombinant human macrophage colony-stimulating factor: resuspend 25 µg of lyophilized rhMCSF in 500 µL of PBS + 0.1% BSA to have stock of 50 µg/mL. Make aliquots of 20 µL. Store at -20°C. Avoid freeze/thaw cycles. Add to a medium at a final concentration of 25 - 50 ng/mL.
16. PBMC-buffer: 2 mM EDTA + 10% FBS. Weight 29.2 mg of EDTA and dissolve it in 45 mL of PBS. Filter-sterilize by using a 0.22 µm PVDF membrane. Add 5 mL of FBS and sonicate for 30 min. Store at 4°C.
17. Complete RPMI medium: add 50 mL of FBS to a 500 mL bottle of RPMI 1640+Glutamax medium. Store at 4°C for up to one month.

18. Sterile 50 mL tubes.
19. Hemocytometer (Recommended: Automated Cell Counter such as Countess from Invitrogen).
20. Cryo tubes.
21. Sterile 384-well assay plates with lids, suitable for fluorescence microscopy.
22. Humidified 37°C incubator maintaining a 5% CO<sub>2</sub> atmosphere.
23. Recommended: 20°C incubator for detachment of macrophages from UpCell plates.

### ***2.3 Cell labelling***

1. RPMI1640, no phenol red.
2. L-glutamine, 200 mM: Make aliquots of 5 mL. Store at -20°C. Then add to a bottle of medium at a final concentration of 2mM.
3. Dimethylsulfoxide (DMSO): store at room temperature in the dark.
4. Fetal bovine serum (FBS): heat-inactivate at 56°C for 30 min. Store at -20°C in 50 mL aliquots.
5. Complete RPMI assay medium: add 50 mL of FBS to a 500 mL bottle of RPMI 1640, no phenol red, supplemented with 2mM glutamine. Store at 4°C for up to one month.
6. MitoTracker Red FM: Dissolved in DMSO at 1 mM and store at -20°C. Add to a medium at a final concentration of 100 nM.
7. Hoechst 33342 (10 mg/mL): Store at 4°C. Add to a medium at a final concentration of 300 ng/mL.
8. Humidified 37°C incubator maintaining a 5% CO<sub>2</sub> atmosphere.
9. Multichannel pipette (16 channels) with adapted tips equipped with filter.
10. Sterile 50 mL polystyrene reservoirs.

### ***2.4 Preparation of bacteria for Infection***

1. RPMI1640, no phenol red.

2. Sterile 15 mL tubes.
3. Spectrophotometer.
4. Cuvette.
5. Water Bath at 37°C.
6. Humidified 37°C incubator maintaining a 5% CO<sub>2</sub> atmosphere.

### ***2.5 Image acquisition and analysis***

1. Laser-based confocal imaging platform: IN Cell Analyzer 6000 (GE Healthcare), Opera High-Content Screening System (Perkin Elmer), or similar instrument adapted to 384-well plates. The instrument should be equipped with 405, 488, 561 and 640 nm excitation lasers as well as 60X objectives.
2. Image analysis software (recommended: Columbus from Perkin Elmer)

### ***2.6 Analysis of Mitochondrial respiration (Seahorse)***

1. Seahorse XFe96 Sensor Cartridges (Agilent).
2. Seahorse XFe96 Cell Culture microplates (Agilent).
3. Seahorse XF Cell Mito Stress Test Kit (Agilent).
4. Seahorse XF Base Medium (Agilent).
5. 100 mM Pyruvate.
6. 2.5 M Glucose.
7. Seahorse XF Calibrant (Agilent).
8. Seahorse XFe96 Flux Analyzer (Agilent) running Wave software.
9. Humidified 37°C incubator maintaining a 5% CO<sub>2</sub> atmosphere.
10. Humidified 37°C non-CO<sub>2</sub> incubator.
11. pH meter (recommended: portable pH meter such as Mettler Toledo F2)
12. Stock compounds of the Seahorse XF Cell Mito Stress Test Kit are prepared as follows: 1) Oligomycin: Add 630 µL of Assay Medium to the tube for a final stock

concentration of 100  $\mu\text{M}$ ; 2) FCCP: Add 720  $\mu\text{L}$  of Assay Medium to the tube for a final stock concentration of 100  $\mu\text{M}$ ; and 3) Rotenone+AntimycinA: Add 540  $\mu\text{L}$  of Assay Medium to the tube for a final stock concentration of 50  $\mu\text{M}$  (*see Note 2*). Gently pipette up and down (>10 times) to solubilize the compounds.

13. Prepare 3 mL of each compound in Assay Medium as follows: 1) Olygomycin: Add 150  $\mu\text{L}$  of Olygomycin 100  $\mu\text{M}$  to 2850  $\mu\text{L}$  of Assay Medium for a 10X concentration of 5  $\mu\text{M}$  (Final concentration in the well: 0.5  $\mu\text{M}$ ); 2) FCCP: Add 150  $\mu\text{L}$  of FCCP 100  $\mu\text{M}$  to 2850  $\mu\text{L}$  of Assay Medium for a 10X concentration of 5  $\mu\text{M}$  (Final concentration in the well: 0.5  $\mu\text{M}$ ); and 3) Rotenone+AntimycinA: Add 300  $\mu\text{L}$  of Rotenone+AntimycinA 50  $\mu\text{M}$  to 2700  $\mu\text{L}$  of Assay Medium for a 10X concentration of 5  $\mu\text{M}$  (Final concentration in the well: 0.5  $\mu\text{M}$ ).

### **3. Methods**

#### ***3.1 Isolation of Human Primary Cells.***

1. Human blood is collected from healthy volunteers (*see Note 3*).
2. Add 15 mL of Ficoll-Paque solution to 50 mL falcon tubes.
3. Dilute one volume of blood in one volume of 1% FBS in PBS.
4. Add slowly 30 mL of diluted blood over the Ficoll-Paque solution, carefully for not mixing both phases.
5. Centrifuge at  $400 \times g$  for 20 min. Set the braking speed of the centrifuge to its minimum value.
6. Collect peripheral blood mononuclear cells (PBMCs), which are located in the white ring at the interface between the plasma (top) and the Ficoll-Paque (bottom). Collect the white layer carefully into new sterile tubes.
7. Wash PBMCs three times by centrifugation at  $400 \times g$  for 10 min at room temperature. Resuspend the pellet in 50 mL 1% FBS in PBS.



8. Pool PBMCs in one 50 mL tube. Count cells using a hemocytometer.
9. Centrifuge PBMCs at  $400 \times g$  for 10 min.
10. Resuspend  $10^7$  total cells in 80  $\mu$ L of ice-cold PBMC-buffer.
11. Add 20  $\mu$ L of Anti-human CD14 antibodies coupled to magnetic beads.
12. Incubate for 15 min at  $4^\circ\text{C}$ .
13. Dilute cells in 2 mL of ice-cold PBMC-buffer.
14. Centrifuge at  $400 \times g$  for 10 min at  $4^\circ\text{C}$  and resuspend the pellet in 0.5 mL of ice-cold PBMC-buffer.
15. Wash the column three times with 3 mL of cold PBMC-buffer.
16. Apply cell suspension onto a column placed in a magnetic field (such as QuadroMACS Separator placed on a MACS MultiStand)
17. Rinse twice with 3 mL of ice-cold PBMC-buffer.
18. Remove the column from the magnetic fields.
19. To elute CD14<sup>+</sup> monocytes, place the column over a new 15 mL sterile tube and firmly flush the column with 5 mL of ice-cold PBMC-buffer.
20. Count cells using a hemocytometer.
21. Centrifuge cells at  $400 \times g$  for 10 min.
22. Resuspend cells to  $2 \times 10^7$  cells/mL in complete RPMI medium containing 10% DMSO.
23. Make aliquots of 1 mL in cryo tubes and store at  $-80^\circ\text{C}$  until the use.

### ***3.2 Cell culture, differentiation and preparation of hMDMs for Infection***

1. 5 days before the assay, defreeze 2 vials of CD14<sup>+</sup> monocytes at  $37^\circ\text{C}$  for 3 min.
2. Transfer 2 mL of cells to 8 mL of pre-warmed complete RPMI medium.
3. Centrifuge cells at  $400 \times g$  for 10 min.

4. Resuspend cells in 18 mL of complete RPMI medium supplemented with 50 ng/mL of rhM-CSF and seed cells in a UpCell 6 well multidish plate, 3mL/well (*see Note 4*).
5. Incubate cells for four days at 37°C, 5% CO<sub>2</sub>.
6. At day 4, replace the medium with fresh RPMI 1640 medium supplemented with 20 ng/mL of rhM-CSF and incubate cells for one more day at 37°C, 5% CO<sub>2</sub>.
7. At day 5, place the UpCell plate 15 min in a 20°C incubator (or at room temperature, *see Note 4*).
8. Gently flush the wells and recover the cells, monitor the detachment process in an inverse microscope.
9. Transfer cells to a 50 mL falcon tube.
10. Add 1 ml of cold D-PBS to each well and gently flush again.
11. Transfer cells to the 50 mL falcon tube of step 9.
12. Centrifuge cells at 400 × g for 10 min.
13. Resuspend in 2 ml complete RPMI medium.
14. Count cells using a hemocytometer.
15. (A) Dilute cells to 3.75 × 10<sup>5</sup> cells/mL (microscopy, mitochondrial dynamics) or (B) 6.25 × 10<sup>5</sup> cells/mL (Seahorse, mitochondrial activity) in complete RPMI medium supplemented with 25ng/mL rhM-CSF (*see Note 5*).
16. (A) Distribute 40 μL of cell solution to 384-well plate (Fig. 2a) to have a final cell density of 15000 cells per well. (B) In the case of the Mitochondrial Respiration assay (Seahorse), plate 80 μL of cell solution to each well of a Seahorse Cell Culture microplate to have a final cell density of 50000 cells per well (Fig. 2b).
17. Incubate plates with hMDMs overnight at 37°C, 5% CO<sub>2</sub>.
18. Turn on the Agilent Seahorse XFe96 Analyzer, and let it warm up overnight.

19. Hydrate a Seahorse XFe96 Sensor Cartridge in Seahorse XF Calibrant at 37 °C in a non-CO<sub>2</sub> incubator overnight.

### ***3.3 Mitochondrial dynamics: mitochondrial labeling of hMDMs prior to infection***

1. Dilute MitoTracker Red FM to a concentration of 100 nM in pre-warmed complete RPMI assay medium.
2. Remove supernatant from plate.
3. Add 40 µL Mitotracker solution onto cells (Fig. 2a).
4. Incubate plate for 30 min at 37°C, 5% CO<sub>2</sub>.
5. Completely remove Mitotracker solution from plate.
6. Carefully wash cells using 50 µL pre-warmed complete RPMI assay medium by up-and-down pipet mixing in 3 times (*see Note 6*).
7. Incubate plate for 5 min at 37°C, 5% CO<sub>2</sub>.
8. Repeat 2 times for step 6 and 7.
9. Replace medium with 50 µL of FBS-free RPMI1640 medium, no phenol red.

### ***3.4 L. pneumophila culture, preparation and infection of hMDMs***

1. The day of the assay, harvest *L. pneumophila* constitutively expressing GFP (*see Note1*) grown for three days at 37 °C on BCYE agar plates supplemented with the corresponding antibiotics.
2. Collect bacteria using 10 µL disposable inoculation loops and dissolve in DPBS to be OD<sub>600</sub> of 2.5 ( $2.2 \times 10^9$  bacteria/mL).
3. Dilute bacteria to be  $3.75 \times 10^6$  bacteria/mL (MOI=10, microscopy) or  $6.25 \times 10^6$  bacteria/mL (MOI=10, Seahorse) in FBS-free RPMI1640 medium, no phenol red.

4. Remove 40  $\mu\text{L}$  of culture medium and add 40  $\mu\text{L}$  of bacterial solution to Mitotracker-stained cells (microscopy, Fig. 2a), or remove 80  $\mu\text{L}$  of culture medium and add 80  $\mu\text{L}$  of bacterial solution to cells (Seahorse, Fig. 2b)
5. Centrifuge plate at  $200 \times g$  for 5 min (*see Note 7*).
6. Incubate plate at  $37^\circ\text{C}$  for 5 min in a water bath (*see Note 8*) followed by incubation of the plate for 25 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ .
7. Remove extracellular bacteria by pipetting thereby mixing 3 times (*see Note 6*).
8. Repeat step 5 two times.
9. (A) Microscopy (mitochondrial dynamics): go to 3.5
10. (B) Seahorse (mitochondrial activity): go to 3.8

### ***3.5 Mitochondrial dynamics: nuclear staining of hMDMs***

1. Dilute Hoechst 33342 to a concentration of 300 ng/mL in pre-warmed complete RPMI assay medium.
2. Remove culture medium and add 50  $\mu\text{L}$  Hoechst 33342 solutions onto infected cells (Fig. 2a).
3. Incubate plate for 30 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ .

### ***3.6 Mitochondrial dynamics: Image acquisition using automatic confocal microscopy***

Images are acquired on an automatic confocal microscopy (*see Note 9*) using 60X objective lens (Fig. 2a). Each condition should be tested in at least quadruplicate wells. Acquire a minimum of 6 fields per well per time point (one to six hours post-infection, p.i.) in order to analyze a minimum of 100 cells per condition and time point.

1. Place the plate into the plate carrier.
2. Establish protocol settings: set up the layout of the plate and the location of the fields that will be acquired per well. Select the 60X objective. Set focus height 1  $\mu\text{m}$ . Select

the excitation laser (Ex) 405 nm and the emission filter (Em) 450 nm for nucleus detection, Ex 488 nm and Em 520 nm for *Legionella*-GFP detection and Ex 561 nm and Em 600 nm for mitochondrial detection. Set the laser power and exposure time to get the intensity around 2000 to 4000. Save the protocol.

3. Acquire images of the plate each hour from 1 to 6 h p.i.

### **3.7 Mitochondrial dynamics: high-content Image analysis**

Scripts for High-content Image analysis can be developed using any object-oriented computer language (C++, Java, Python, etc). We recommend using dedicated software that can manage predefined detection algorithms, such as Columbus image analysis software (PerkinElmer) or IN Cell Investigator (GE Healthcare). Alternatively, open-source software such as Image J (<http://www.imageJ.net>), ICY (<http://www.icy.bioimageanalysis.org/>) or Cell Profiler (<http://www.cellprofiler.org>) can also be used for image analysis. Herein we describe the script used in the Columbus image analysis software to perform the High-content Image analysis of mitochondrial morphology.

1. Import data.
2. Segment nuclei and cytoplasm by Hoechst signals (Fig. 3b).
3. Segment intracellular GFP-expressing *L. pneumophila* by GFP signals (Fig. 3b).
4. Sub-populate infected and bystander non-infected cells.
5. Calculate texture properties by Mitotracker signals (Fig. 3b): The SER edge and ridge signal, normalized by intensity and with parameter set to 0.05  $\mu\text{m}$ , was defined at the single cell level in order to apply for the criteria of sub-population of cells with elongated and fragmented mitochondria (Fig. 3c and 3d).
6. Sub-populate cells with elongated and fragmented mitochondria.
7. Formulate the percentage of infected cells.

8. Formulate the percentage of cells with fragmented mitochondria.
9. Formulate the percentage of cells with elongated mitochondria.
10. Save image script.
11. Run batch analysis.
12. Export data to Excel.

### ***3.8 Mitochondrial activity: analysis of mitochondrial respiration (Seahorse)***

1. The day of the assay, but before infection of hMDMs with *Legionella* (3.4.4), prepare the Seahorse assay (following steps from 3.8.2 to 3.8.7)
2. Prepare the Assay Medium by supplementing Seahorse XF Base Medium with 1 mM pyruvate and 10 mM glucose.
3. Warm the Assay Medium to 37°C, adjust the pH to 7.4 with 0.1 N NaOH and keep at 37°C until use (*see Note 10*).
4. Allow the compounds of the Seahorse XF Cell Mito Stress Test Kit to warm to room temperature in the sealed bag for approximately 15 minutes and prepare the compounds of the Seahorse XF Cell Mito Stress Test Kit as explained in section 2.6.
5. Load compounds in the ports of the Seahorse XFe96 Sensor Cartridges (from 3.2.19) using a 10-100 µL multichannel pipette and the corresponding loading guides as follows: Port A) Add 20 µL of 10X Olygomycin (from 3.8.6); Port B) Add 22 µL of 10X FCCP (from 3.8.6); Port C) Add 25 µL of 10X Rotenone+AntimycinA (from 3.8.6); and Port D) Add 28 µL of Assay Medium (*see Note 11*).
6. Leave the Sensor Cartridge in the 37°C non-CO<sub>2</sub> incubator until use.
7. Design the assay file by specifying the Blank wells (no hMDMs, use the borders of the plate for these controls), the non-infected hMDMs samples and the *Legionella*-infected samples (all recommended by quadruplicate), as well as the routine for port injections and Mix-Wait-Measure cycles (*see Note 12*).

8. Remove Seahorse XF Cell Culture microplate with infected hMDMs (from 3.4.10) from the 37°C-5%CO<sub>2</sub> incubator and change the cell culture medium in the Cell Culture microplate to 180 µL of warmed Assay Medium (37°C) using a multichannel pipette, and place the Cell Culture microplate into a 37°C non-CO<sub>2</sub> incubator for 45 minutes prior to the assay (*see Note 13*).
9. Run the assay by opening the assay file and clicking “Run”.
10. Place the calibration plate with the loaded Sensor Cartridge (Fig. 2b) on the instrument tray, and click “Continue”. Calibration takes approximately 15-30 minutes (*see Note 14*).
11. When prompted, replace the calibration plate with the Cell Culture microplate (from 3.8.11) and then click “Start”.
12. For the analysis of the results of the Seahorse XF Mito Stress Test, the OCR is automatically calculated and displayed in a kinetic graph showing the values after each injection (Fig. 4a). To interpret this kinetic graph, OCR values after each injection correspond to several different mitochondrial respiration parameters (Fig. 4b). Moreover, a summary of mitochondrial respiration parameters during *Legionella* infection of hMDMs can be automatically obtained by exporting the assay results in an Excel file and using it on the Seahorse XF Stress Test Report Generator (*see Note 15*).

#### 4. Notes

1. *L. pneumophila* strains used to analyze mitochondrial dynamics should constitutively express GFP. This is normally done by transformation of *L. pneumophila* with a plasmid harboring GFP, such as pNT28 [15]. By comparing WT-GFP infection with isogenic mutants expressing GFP we can study in parallel the effect of different *Legionella* genes on the modulation of mitochondrial dynamics during infection. In addition, the use of other GFP-expressing *Legionella* species permits to measure the

impact on mitochondrial dynamics of the infection by different *Legionella* species in human macrophages.

2. Use compounds the same day they are reconstituted and do not refreeze.
3. Human blood was collected by the French National Blood Service (EFS) from healthy volunteers after obtaining their informed consent and under the ethical rules established by the Ethics and Professional Conduct Committee of the EFS, which imply anonymity about the volunteers' gender, sex or age.
4. UpCell plates have a temperature-responsive surface that support non-enzymatic harvesting of adherent cells for preservation of cell viability and surface proteins. As the hydrophobicity of the UpCell surface changes with the external temperature, cells detach with no need for dissociation enzymes nor physical scraping, resulting in high hMDM viability. Best results in hMDM detachment are obtained with 15 min incubation in a 20°C incubator, however longer times at room temperature (>20min) also work well. To avoid unintentional cell detachment, it is very important to pre-warm M-CSF-containing medium added to 37°C .
5. Most of the methods and steps are shared for the mitochondrial dynamics and the mitochondrial activity assay, however when different volumes or approaches are used, A and B letters will be used as follows: (A) Microscopy, mitochondrial dynamics; and (B) Seahorse, mitochondrial respiration.
6. Do not touch bottom of the well and do not make bubbles. To avoid cell detachment, we recommend leaving a residual volume of 10 µL during the washes.
7. The infection is synchronized by centrifugation followed by 5 min incubation in a 37°C water bath.



8. 5 min incubation in a 37°C water bath is critical for uniform bacterial infection of cells. Plates float on the water without any supplementary device (leave and take them carefully).
9. Automated confocal microscopy: IN Cell Analyzer 6000/200 (GE Healthcare), Opera/Operetta (Perkin Elmer), BD Pathway 855/435 (BD Biosciences), ImageXpress Ultra/Micro (Molecular Devices), CellomicsArrayScan (Thermo Scientific), ScanR (Olympus), Yokogawa CV7000/8000 (Wako Automation).
10. By using a portable pH meter it is possible to measure and adjust the pH under the hood.
11. Each series of ports must contain the same volume (For example, all A ports must be filled with the same volume; all B ports must be filled with the same volume, etc). All wells, including Blank wells, need to have solution loaded in all the ports to ensure proper injection in all wells. If a port is not used, fill it with Assay Medium. The loading guides come with the Sensor Cartridge and help in the loading of the compounds into the ports by guiding the multichannel pipette to load the right ports.
12. The typical Mix-Wait-Measure times are 3 min – 2 min – 3 min. In general, 3 basal measurements are taken prior to the first injection, and then 3 measurements are taken after each injection.
13. Thus 1h post-infection is the earliest time point to measure mitochondrial respiration.
14. Important: remove the Cartridge lid and verify the correct plate orientation. Have in mind the calibration time (15-30 min) in order to adjust the first measurements to the desired time post-infection.

15. The Seahorse XF Stress Test Report Generator is freely available, after registration, at [https://www.agilent.com/en-us/products/cell-analysis-\(seahorse\)/xf-cell-mito-stress-test-report-generator](https://www.agilent.com/en-us/products/cell-analysis-(seahorse)/xf-cell-mito-stress-test-report-generator).

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## Figure Captions

**Figure 1. Mitochondrial dynamics and bioenergetics.** Respiratory capacity and mitochondrial morphology are functionally related.

**Figure 2. Work-flow of cell-based assays.** Work-flow of the measurement of mitochondrial dynamics using microscopy-based assays **(a)** and of mitochondrial activity using the Seahorse assay **(b)**.

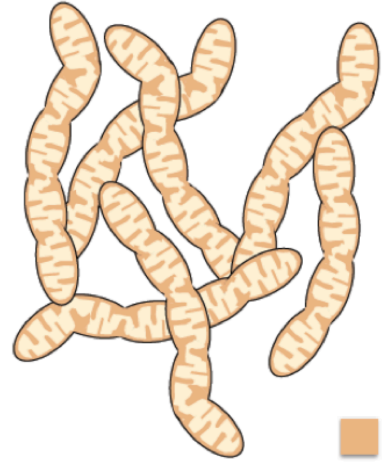
**Figure 3. Mitochondrial texture properties and image analysis.** **(a)** Typical confocal images of elongated and fragmented mitochondria and Edge / Ridge filtered images. Blue, nuclei; Red, mitochondria; MT, mitochondria **(b)** Image analysis scheme for the cell segmentation and SER texture analysis of mitochondria **(c)** SER Edge and Ridge properties at the single cell level. Each dot represents a cell ( $n > 13$  per condition).  $***p \leq 0.001$  (Mann-Whitney t test). **(d)** Example of cut-off values to sub-populate mitochondrial phenotypes.

**Figure 4. Mitochondrial respiration assay (Seahorse) of hMDMs during *Legionella* infection.** **(a)** hMDMs were un-infected or infected for 6 h with *L. pneumophila* JR32 wild-type (WT) or its isogenic *icmT* deficient mutant. Then, the mitochondrial respiration assay was performed by measuring OCR during basal conditions and after Olygomycin injection, FCCP injection and Rotenone+AntimycinA injection. The kinetic graph shown is the output of the device. **(b)** Bioenergetic profiles of the key parameters obtained during the mitochondrial respiration assay using the Seahorse XF Mitostress kit (Source: Agilent).

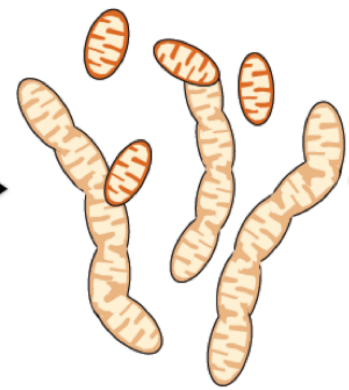
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**Hyperfused network**



**Dynamic mitochondria**

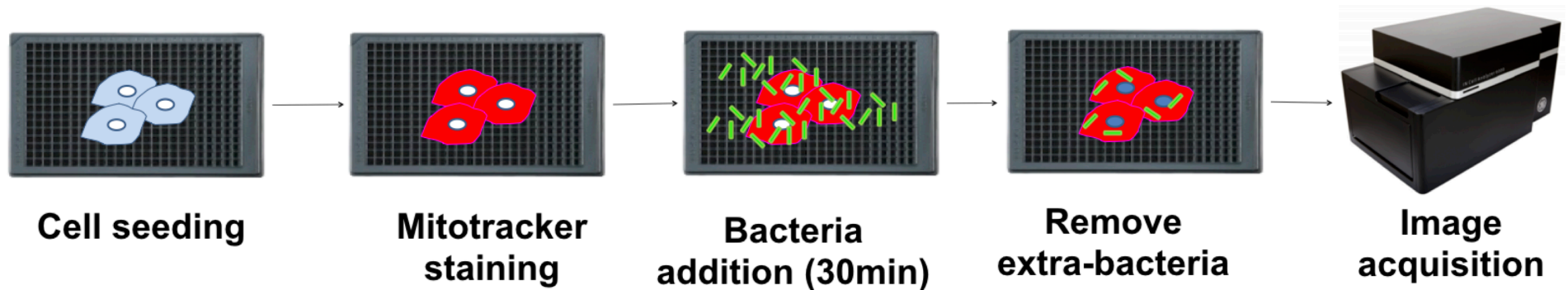


**Fragmented mitochondria**

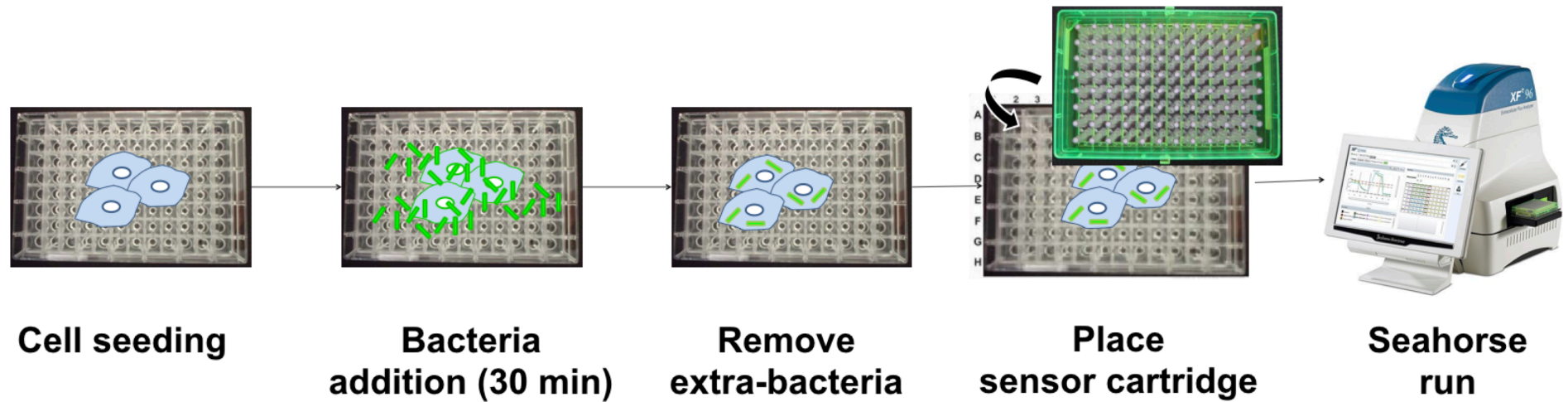


■ High respiratory capacity ■ Low respiratory capacity

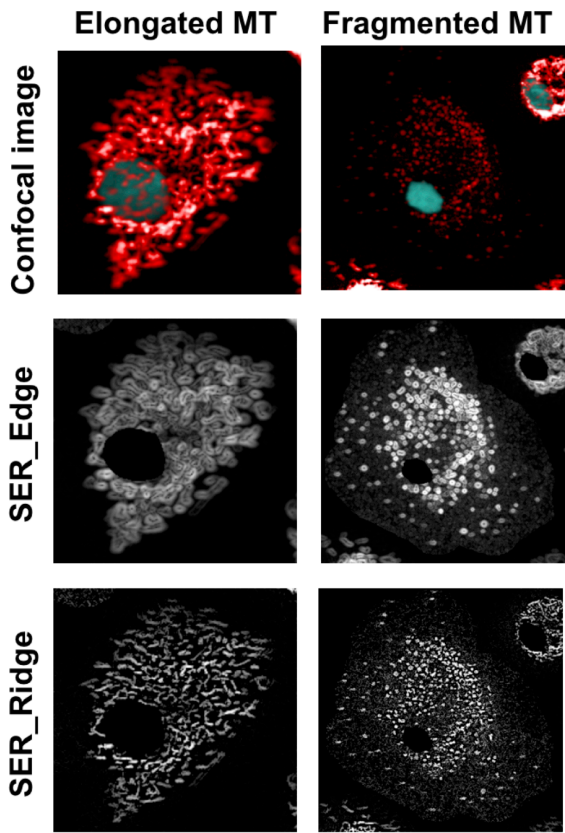
## a. Mitochondrial dynamics (microscopy-based assay)



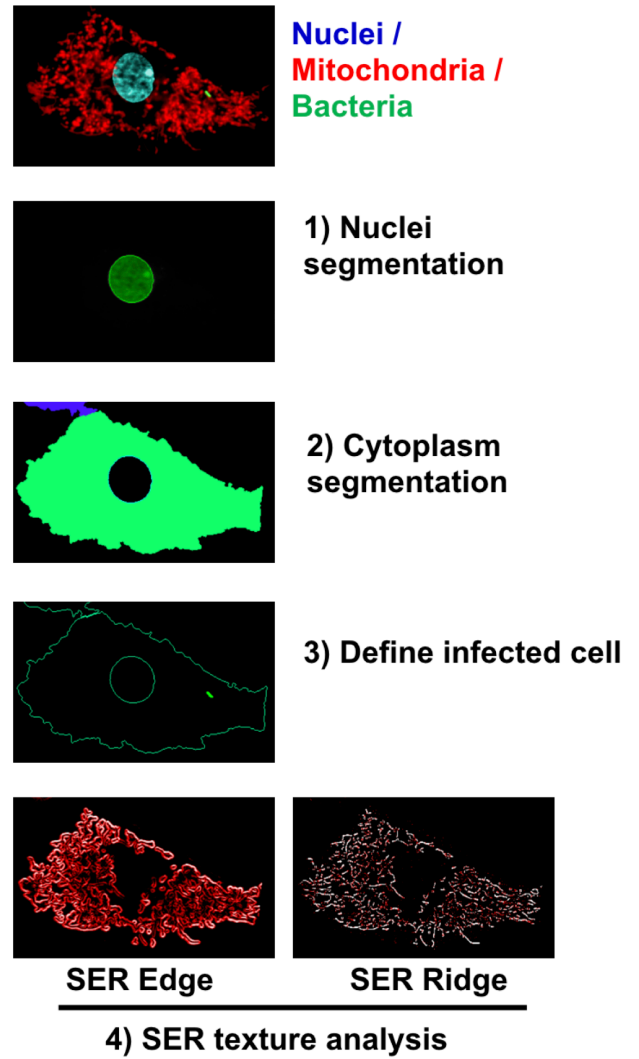
## b. Mitochondrial activity (Seahorse assay)



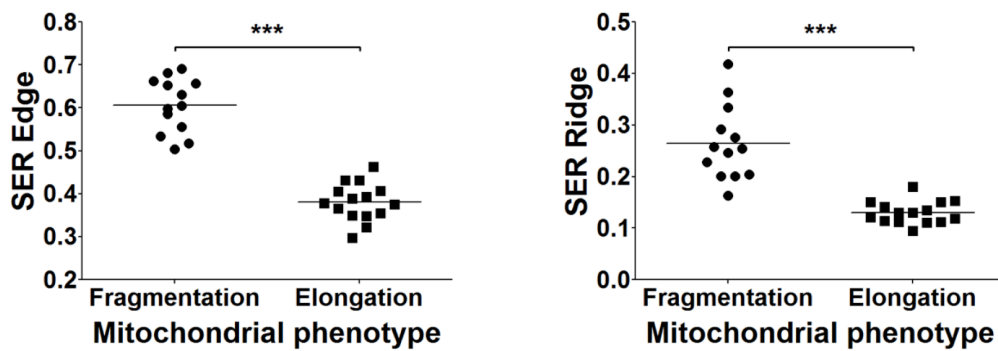
**a** Typical confocal images and SER filtered images



**b** Image analysis scheme



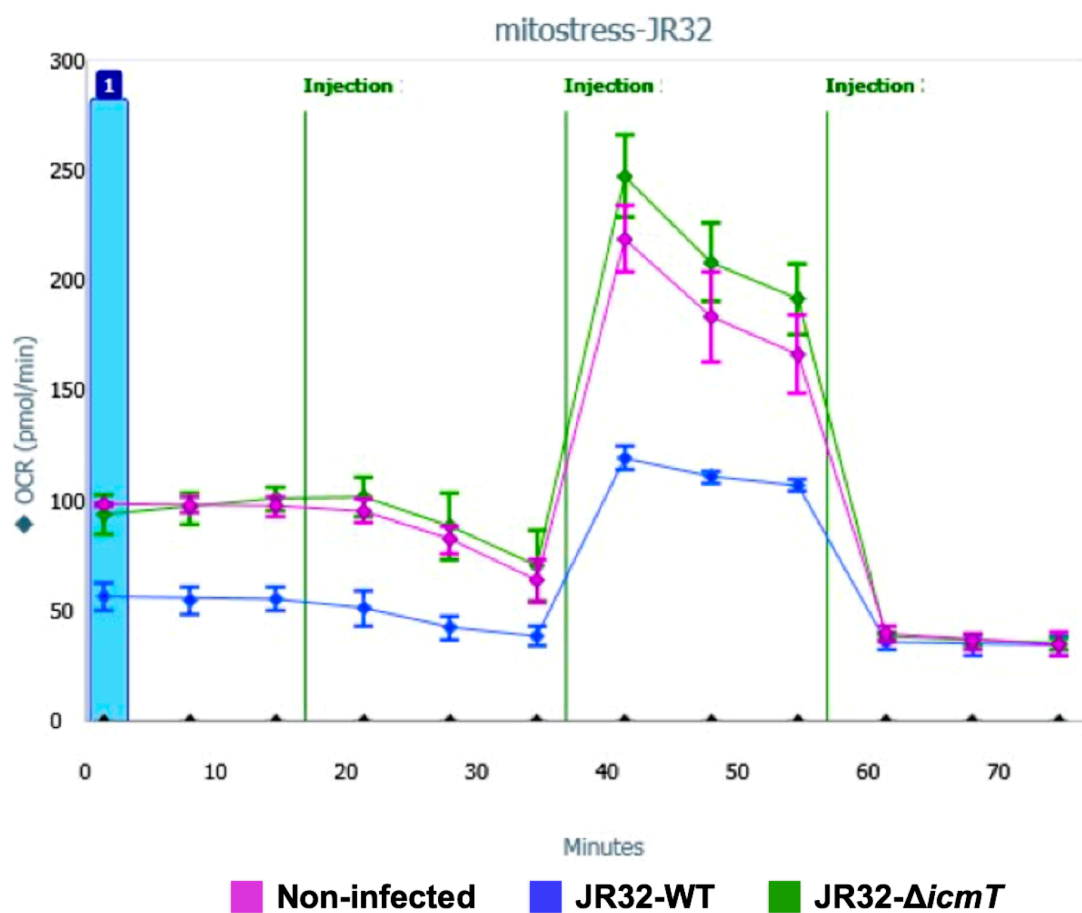
**c** Single cell analysis



**d**

SER feature	Mitochondrial phenotype	
	Fragmentation	Elongation
Edge	$\geq 0.5$	$\leq 0.5$
Ridge	$\geq 0.2$	$\leq 0.2$

a



b

