Video Article

Quantification of Cytosolic vs. Vacuolar *Salmonella* in Primary Macrophages by Differential Permeabilization

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Abstract

Intracellular bacterial pathogens can replicate in the cytosol or in specialized pathogen-containing vacuoles (PCVs). To reach the cytosol, bacteria like *Shigella flexneri* and *Francisella novicida* need to induce the rupture of the phagosome. In contrast, *Salmonella typhimurium* replicates in a vacuolar compartment, known as *Salmonella*-containing vacuole (SCV). However certain mutants of *Salmonella* fail to maintain SCV integrity and are thus released into the cytosol. The percentage of cytosolic vs. vacuolar bacteria on the level of single bacteria can be measured by differential permeabilization, also known as phagosome-protection assay. The approach makes use of the property of detergent digitonin to selectively bind cholesterol. Since the plasma membrane contains more cholesterol than other cellular membranes, digitonin can be used to selectively permeabilize the plasma membrane while leaving intracellular membranes intact. In brief, following infection with the pathogen expressing a fluorescent marker protein (*e.g.* mCherry among others), the plasma membrane of host cells is permeabilized with a short incubation in digitonin containing buffer. Cells are then washed and incubated with a primary antibody (coupled to a fluorophore of choice) directed against the bacterium of choice (*e.g.* anti-*Salmonella*-FITC) and washed again. If unmarked bacteria are used, an additional step can be done, in which all membranes are permeabilized and all bacteria stained with a corresponding antibody. Following the staining, the percentage of vacuolar and cytosolic bacteria can be quantified by FACS or microscopy by counting single or double-positive events. Here we provide experimental details for use of this technique with the bacterium *Salmonella typhimurium*. The advantage of this assay is that, in contrast to other assay, it provides a quantification on the level of single bacteria, and if analyzed by microscopy provides the exact number of cytosolic and vacuolar bacteria in a given cell.

Video Link

The video component of this article can be found at https://www.jove.com/video/52960/

Introduction

Intracellular bacterial pathogens replicate either directly in the host cell cytosol, or in specialized vacuolar compartments[1]. During the initial stage of the infection most pathogens get internalized either by phagocytosis by specialized cells (such as macrophages) or by actively promoting their own uptake into non-phagocytic cells. In phagocytic cells, the phagosome normally fuses with lysosomes to form a degradative compartment, where the phagocytosed particles are digested. Specialized cytosolic bacteria, such as *Francisella novicida* or *Shigella flexneri*, escape phagosomal degradation by inducing the rupture of the phagosome and subsequently escape into the cytosol[2,3]. This requires virulence-associated mechanism such as the *Francisella* Pathogenicity Island (FPI) or the *Shigella flexneri* T3SS, which injects effector proteins that promote vacuolar rupture[2-4]. The host cell cytosol features a number of conserved pattern recognition receptors that normally recognize the presence of pathogens and induce innate immune signaling[5]. In addition, xenophagy, an anti-microbial form of autophagy can degrade bacteria that enter the cytosol. Cytosolic bacteria are normally well adapted to blunt or circumvent these responses using different strategies. For example, *Francisella* modifies its LPS to avoid host recognition and *Shigella* prevents autophagy recruitment using secreted effector proteins[6,7].

Another strategy to escape lysosomal degradation is employed by the model vacuolar pathogen *Salmonella enterica* serovar Typhimurium, thereafter referred to as *Salmonella typhimurium*. *Salmonella* uses a T3SS to inject effectors that remodel the initial phagosome into its intracellular niche, the *Salmonella*-containing vacuole (SCV) [8,9]. Continuous manipulation of host pathways is necessary to maintain this vacuole by recruiting lipids and other nutrients to the vacuole. Indeed, a sifA mutant of *Salmonella* fails to maintain vacuolar stability, and enters the cytosol of host cells within hours after infection, which results in activation of innate immune pathways and autophagy recruitment[8]. Vacuolar escape of *Salmonella* varies depending on the cell type that is studies, and several reports have shown that in epithelial cells even WT *Salmonella* can escape the SCV and hyperreplicate in the cytosol[10]. Recent reports show that innate immune detection of vacuolar pathogens also depends on the ability of the host to recognize and destabilize pathogen-containing vacuoles (PCVs) [11-14].

Given that both the host or bacteria genotype can affect the distribution of intracellular bacteria between the vacuolar and cytosolic compartment, it is necessary to quantify the number of vacuolar vs. cytosolic bacteria. Since, in most experimental setups host cells are infected with more than one bacterium, and subsequently can harbor several bacteria in different subcellular compartments, a technique is needed that allows

quantification on the level of single bacteria. Differential permeabilization (also known as phagosomal protection assay) provides this resolution [2,4,10,12]. The assay is based on selective permeabilization of the host cell plasma membrane with the detergent digitonin, which leaves vacuolar membranes intact and thus allows selective staining of cytosolic bacteria with antibodies. Here we provide two protocols for the quantification of cytosolic and vacuolar *Salmonella typhimurium* using differential permeabilization. The principle of this method is described in **Figure 1**: Bone-marrow derived macrophages (BMDMs) are infected with stationary phase mCherry-expressing *Salmonella*. Stationary phase *Salmonella* need to be used because they downregulate the expression of the SPI-1 T3SS, whose activity would otherwise be recognized by the NLRC4 inflammasome and induce rapid cell death (pyroptosis) of the BMDMs[15]. Following the infection cells are washed and treated with 50 µg/ml of digitonin for 1 minute. Cells are washed again immediately and incubated with anti-*Salmonella* antibodies coupled to FITC to mark bacteria with access to the cytosol. After an additional washing step cells are lysed and the percentage of mCherry+ (vacuolar) and FITC+ / mCherry+ (cytosolic) bacteria is determined by FACS. We also report an adaptation of this method that can be used if no fluorescent protein-expressing strains are available (**Figure 2**). Additional steps are introduced after the FITC labeling in which cells are fixed and completely permeabilized. Thereafter all bacteria are stained with anti-*Salmonella* antibodies and corresponding secondary antibodies. Detection is then done by microscopy instead of FACS analysis.

Protocol

1. Digitonin Assay with mCherry-expressing *Salmonella* (FACS-based Analysis)

1. Preparing bacteria
   Note: *Salmonella* wild-type SL1344 (Streptomycin resistant) expressing mCherry from a plasmid (pFPV encoding mCherry under the rpsM promoter) is used in this assay. Bacterial phagocytosis and proliferation where not altered by the constitutive expression of mCherry (data not shown) [16].
   1. Inoculate the strain 1 day before the infection in 3 ml of LB medium supplemented with Streptomycin (90 µg/ml) and Ampicillin (100 µg/ml, mCherry expressing vector) in a shaker at 37 °C O/N.

2. Plating cells for infection.
   1. Seed wild-type BMDMs in a 24 well plate at a density of 1.25 x 10^5 cells/well in 1 ml of primary macrophage medium (DMEM supplemented with 10% Fetal Calf Serum (FCS, heat-inactivated, 56 °C, 30 min), 1% HEPES, 1% Non-essential amino acids (NEAA), 1% L-Glutamine and 10% L929 supernatant (conditioned medium from macrophage colony-stimulating factor (M-CSF) producing L929 cells). Seed wells according to **Table 1** to account for the individual conditions. Add coverslips to the wells used as controls (**Table 1**).
   2. Incubate the cells overnight in an incubator at 37 °C and 5% CO_2.

			Permeabilization		Staining		
	Glass coverslip	Salmonella	Digitonin	Saponin	anti-Salmonella	anti-Calnexin	anti-PDI
A1 (sample)	-/+*	+	+		+		
A2 (sample)	-/+*	+	+		+		
A3 (sample)	-/+*	+	+		+		
A4 (no permeabilization control)	-/+*	+			+		
A5 (complete permeabilization control)	-/+*	+		+	+		
B1 (stained for calnexin)	+					+	
B2 (stained for calnexin)	+		+			+	
B3 (stained for calnexin)	+			+		+	
C1 (stained for PDI)	+						+
C2 (stained for PDI)	+		+				+
C3 (stained for PDI)	+			+			+

Table 1: Plating scheme for bone-marrow derived macrophages (BMDMs) in a 24-well format for subsequent infection with Salmonella, permeabilization, and staining. *Depending on whether protocol 1 or protocol 2 is done.

3. Preparing the bacteria for infection.

1. The next day, determine the concentration of *Salmonella* in the overnight culture by diluting the culture 1:10 in PBS and measuring the OD_{600} against a PBS only control. This ensures that the OD_{600} is measured in the linear range of the spectrophotometer.
2. Calculate the concentration of *Salmonella* per ml. An OD600 of 1 corresponds to 1 x 10^9 bacteria.
3. Dilute the bacteria in macrophage medium to reach a *Salmonella* concentration of 1.25 x 10^6 cells/ml.

4. Infection of cells with *Salmonella*.
   1. Remove medium from BMDMs. Add 1 ml of macrophage medium to uninfected control wells (B1-B3, C1-C3). Add 1 ml of *Salmonella* suspension to wells A1 - A5 to reach a multiplicity of infection (m.o.i.) of 10 bacteria per cell.
   2. Centrifuge the plate for 15 min at 300 x g at 37 °C to synchronize the infection. Transfer the plate in an incubator at 37 °C and 5% CO_2.

5. Killing extracellular *Salmonella* with Gentamicin.
   1. At 1 hr post-infection, remove plate from incubator and add 0.1 ml of macrophage medium containing 0.1 mg/ml Gentamicin to kill extracellular bacteria. Add Gentamicin to all wells, even to uninfected controls, to ensure that all cells are treated the same way. Transfer the plate in an incubator at 37 °C and 5% CO_2.

6. Washing the cells.
   1. At 2 hr post infection, remove plate from incubator and wash all wells 2x with 1 ml of plain DMEM (pre-warmed to 37 °C) and replace it with macrophage medium (pre-warmed to 37 °C) containing 10 µg/ml Gentamycin to prevent growth of any remaining extracellular bacteria. Transfer the plate in an incubator at 37 °C and 5% CO_2.

7. Preparing fresh buffers with detergents and antibodies.
   1. Just before the desired time point of analysis, prepare a fresh stock solution of digitonin at 1 mg/ml in KHM Buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$, pH 7.3). Filter the stock and dilute in KHM buffer to a working concentration of 50 µg/ml of digitonin. In addition, prepare a solution of 0.1% Saponin in KHM Buffer, anti-*Salmonella* antibody coupled to FITC (CSA-1-FITC) at 1/500, anti-calnexin at 1/100 or anti-PDI at 1/100 in KHM Buffer supplemented with 3% BSA.

8. Cell permeabilization.
   1. Remove plate from incubator and wash wells 3x with 0.5 ml of KHM Buffer (pre-warmed to 37 °C). Remove KHM Buffer and add 0.25 ml plain KHM Buffer to wells A4 and B1/C1, KHM Buffer with digitonin to wells A1 - A3 and B2/C2 or KHM Buffer with saponin to wells A5 and B3/C3 (according to **Table 1**). Incubate for exactly 1 min at RT and immediately wash all wells 3x with 0.5 ml of KHM Buffer (pre-warmed to 37 °C).

9. Primary antibody staining.
   1. Remove the KHM Buffer and add the primary antibody solutions (Goat anti *Salmonella*-FITC, 250 µl/well, 0.1 µg/ml) to the appropriate wells (**Figure 1**, **Table 1**). Incubate the plate for 15 min in an incubator at 37 °C and 5% CO_2.
   2. Wash the cells 1x with 1 mL PBS.

10. Infected Cells (wells A1 - A5): FACS analysis
    1. Wash cells 3x with PBS and add 0.5 mL of ice-cold PBS containing 0.1% Triton. Transfer to a 5 mL FACS tube with cell strainer cap to break up cell aggregates. Keep samples on ice.
    2. Analyze the samples of a FACS. Using the fully permeabilized controls, set the gate for total bacteria based on forward and side scatter (FSC/SSC) first, and the mCherry signal (610 nm ± 20 nm filter).
    3. Next, analyze the FITC signal in the total bacterial population using the fully permeabilized and not permeabilized controls to set the gates for cytosolic bacteria (FITC+, mCherry+) and vacuolar bacteria (FITC-, mCherry+) (**Figure 3**).
    4. With the gates set, analyze the samples and determine the percentage of cytosolic vs. vacuolar bacteria using FlowJo software according to manufacturer's protocol.

11. Uninfected permeabilization controls (wells B1 - B3, C1 - C3): microscopy
    1. Fixation
       1. Remove PBS and add 250 µl of PFA 4% in PBS. Incubate for 10 min at 37 °C.
       2. Wash wells 2x with 1 ml of PBS and add 250 µl of 0.1 M glycine in PBS for 10 min to quench the fixative. Wash wells 2x with 1 ml of PBS.

    2. Secondary antibody staining.
       1. Stain permeabilization controls for 1 hr at RT with appropriate secondary antibodies coupled to fluorophores in PBS supplemented with 0.1% Saponin and 3% BSA to fully permeabilize cells.
       2. Wash coverslips 3x with 1 ml of PBS and mount the coverslips for analysis in mounting medium with DAPI (1.5 µg/ml).

    3. Microscopy analysis.
       1. Analyze the coverslips with the controls at a 40X or 63X magnification using a confocal fluorescence microscope. If the permeabilization has worked, there should be no staining for non-permeabilized cells, Calnexin staining for both digitonin- and Saponin-permeabilized cells and PDI staining only for saponin-permeabilized cells (**Figure 4**).

2. Digitonin Assay with Unlabeled Salmonella (Microscopy-based Analysis)

1. Preparing bacteria for O/N cultures.
    1. Inoculate unlabeled *Salmonella* wild-type SL1344 (Streptomycin resistant) 1 day before the infection in 3 ml of LB medium supplemented with Streptomycin (90 µg/ml) in a shaker at 37 °C O/N.
    2. Plating cells for infection.
        1. Seed BMDMs in a 24 well plate containing sterile glass coverslips at a density of 1.25 x 10^5 cells/well in 1 ml of macrophage medium (DMEM supplemented with 10% Fetal Calf Serum (FCS, de-complemented, 56 °C, 30 min), 1% HEPES, 1% L-Non-essential amino acids (NEAA), 1% L-Glutamine and 10% L929 supernatant). Seed wells according to **Table 1** to account for the individual conditions.
        2. Incubate the cells O/N in an incubator at 37 °C and 5% CO_2.

    3. Preparing the bacteria for infection.
        1. The next day, determine the concentration of *Salmonella* in the O/N culture by diluting the culture 1:10 in PBS and measuring the OD_{600} against a PBS only control. This ensures that the OD_{600} is measured in the linear range of the spectrophotometer.
        2. Determine the concentration of *Salmonella* per milliliter. An OD_{600} of 1 corresponds to 1 x 10^9 bacteria.
        3. Dilute the bacteria in macrophage medium to reach a *Salmonella* concentration of 1.25 x 10^6 cells/ml.

    4. Infection of cells with *Salmonella*.
        1. Remove medium from all wells. Add 1 ml of plain macrophage medium to uninfected control wells (B1 - B3, C1 - C3). Add 1 ml of *Salmonella* suspension to wells A1 - A5 to reach a multiplicity of infection (m.o.i.) of 10 bacteria per cell.
        2. Centrifuge the plate for 15 min at 300 x g at 37 °C to synchronize the infection. Transfer the plate in an incubator at 37 °C and 5% CO_2.

    5. Killing extracellular *Salmonella* with Gentamycin.
        1. At 1 hr post-infection, remove plate from incubator and add 0.1 ml of macrophage medium containing 1 mg/ml Gentamycin to kill extracellular bacteria to all wells. Transfer the plate in an incubator at 37 °C and 5% CO_2.

    6. Washing the cells.
        1. At 2 hr post-infection, remove plate from incubator and wash each well 3x with 1 ml of fresh macrophage medium containing 10 µg/ml Gentamycin to prevent growth of any remaining extracellular bacteria. Transfer the plate in an incubator at 37°C and 5% CO_2.

    7. Preparing fresh buffers with detergents and antibodies.
        1. Just before the desired time point of analysis, prepare a fresh stock solution of digitonin at 1 mg/ml in KHM Buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$, pH 7.3). Filter the stock and dilute in KHM Buffer to a working concentration of 50 µg/ml of digitonin.
        2. In addition, prepare a solution of 0.1% Saponin in KHM Buffer, anti-*Salmonella*-FITC (CSA-1, 0.1 µg/ml), anti-calnexin at 1/100 or anti-PDI at 1/100 in KHM Buffer supplemented with 3% BSA (see materials table for antibody concentration).

    8. Cell permeabilization.
        1. Remove plate from incubator and wash wells 3x with 0.5 ml of KHM Buffer (pre-warmed to 37 °C). Remove KHM Buffer and add 0.25 ml plain KHM Buffer to wells A4 and B1/C1, KHM Buffer with digitonin to wells A1 - A3 and B2/C2 or KHM Buffer with saponin to wells A5 and B3/C3 (according to **Table 1**). Incubate for exactly 1 min at RT and immediately wash all wells 3x with 0.5 ml of KHM Buffer (pre-warmed to 37 °C).

    9. Primary antibody staining.
        1. Remove the KHM Buffer and add the primary antibody solutions (Goat anti *Salmonella* CSA1-FITC, 250 µl/well, 0.1 µg/ml) to the appropriate wells (**Figure 2**, **Table 1**). Incubate the plate for 15 min in an incubator at 37 °C and 5% CO_2. Wash 3x with 1 ml PBS.

    10. Fixation.
        1. Remove PBS and add 250 µl of PFA 4% in PBS. Incubate for 10 min at 37 °C.
        2. Wash wells 2x with 1 ml of PBS and add 250 µl of 0.1 M Glycine in PBS for 10 min to quench the fixative. Wash wells 2x with 1 ml of PBS.

    11. Total *Salmonella* staining of infected samples.
        1. Wash coverslips 3x with PBS with 0.1% Saponin/3% BSA and incubate the samples with an anti-CSA1 antibody (Goat anti-CSA1, 0.1 µg/ml, mouse anti-LPS works as well, 0.1 µg/ml) for 1 hr at RT in PBS with 0.1% Saponin/3% BSA.
        2. Wash 3x in PBS with 0.1% Saponin/3% BSA. Incubate your samples with a secondary anti-Goat antibody coupled to fluorophore of choice in PBS with 0.1% Saponin/3% BSA for 30 min at RT in the dark.
        3. Wash coverslips 3x with 1 ml of PBS and mount the coverslips for analysis in mounting medium with DAPI (1.5 µg/ml).

    12. Microscopy analysis.
        1. Acquire data with a confocal microscope by counting cytosolic *Salmonella* (anti-*Salmonella*-FITC positive) and total *Salmonella* (double positive) (**Figure 5**). Permeabilization controls should show: no staining for non-permeabilized cells, Calnexin staining for digitonin-permeabilized cells and PDI staining for saponin-permeabilized cells (**Figure 4**). Wells A4 and A5 will serve as internal controls for the *Salmonella* staining.

Representative Results

Figure 1 and **Figure 2** show the schematics of the digitonin assay described in protocol 1 and protocol 2 illustrating the critical steps in the protocol and the results obtained. **Figure 3** shows typical FACS results. Positive and negative controls are used to set the gates for mCherry+/FITC- bacteria (vacuolar *Salmonella*) and mCherry+/FITC+ bacteria (cytosolic *Salmonella*). Based on these gates the percentage of cytosolic and vacuolar bacteria can be determined in the experimental samples. In this example we have compared wild-type and ΔsifA *Salmonella*. **Figure 3** shows representative data obtained with mCherry^{+} *Salmonella typhimurium* wild-type and a ΔsifA mutant in bone marrow-derived murine macrophages. **Figure 4** shows the usual Calnexin (**A**) and PDI (**B**) staining that is obtained in permeabilization controls treated with digitonin or saponin. Since Calnexin is an ER membrane protein, Calnexin staining can be seen throughout the cytosol and around the nucleus in both digitonin- and saponin-permeabilized cells. In digitonin permeabilized controls no PDI staining (ER lumen) is visible, while staining can be observed in saponin-permeabilized cells. **Figure 5A** shows anti-*Salmonella* staining result in cells that have been permeabilized with digitonin. Total bacteria are in red, while cytosolic bacteria are in green (stained with anti-*Salmonella*-FITC). The FITC-positive population are *Salmonella* with access to the cytosol, while the FITC-negative population are bacteria that were residing in an intact *Salmonella*-containing vacuole (SCV) and were therefore protected from *Salmonella*-FITC labeling. We have also compared wild-type *Salmonella* to a ΔsifA mutant strain. Since sifA is necessary for *Salmonella* to maintain SCV integrity an increased percentage of *Salmonella* is cytosolic. In **Figure 5B** we show a control, in which the cells were not digitonin permeabilized before adding anti-*Salmonella*-FITC. Accordingly, no FITC-positive bacteria can be seen.

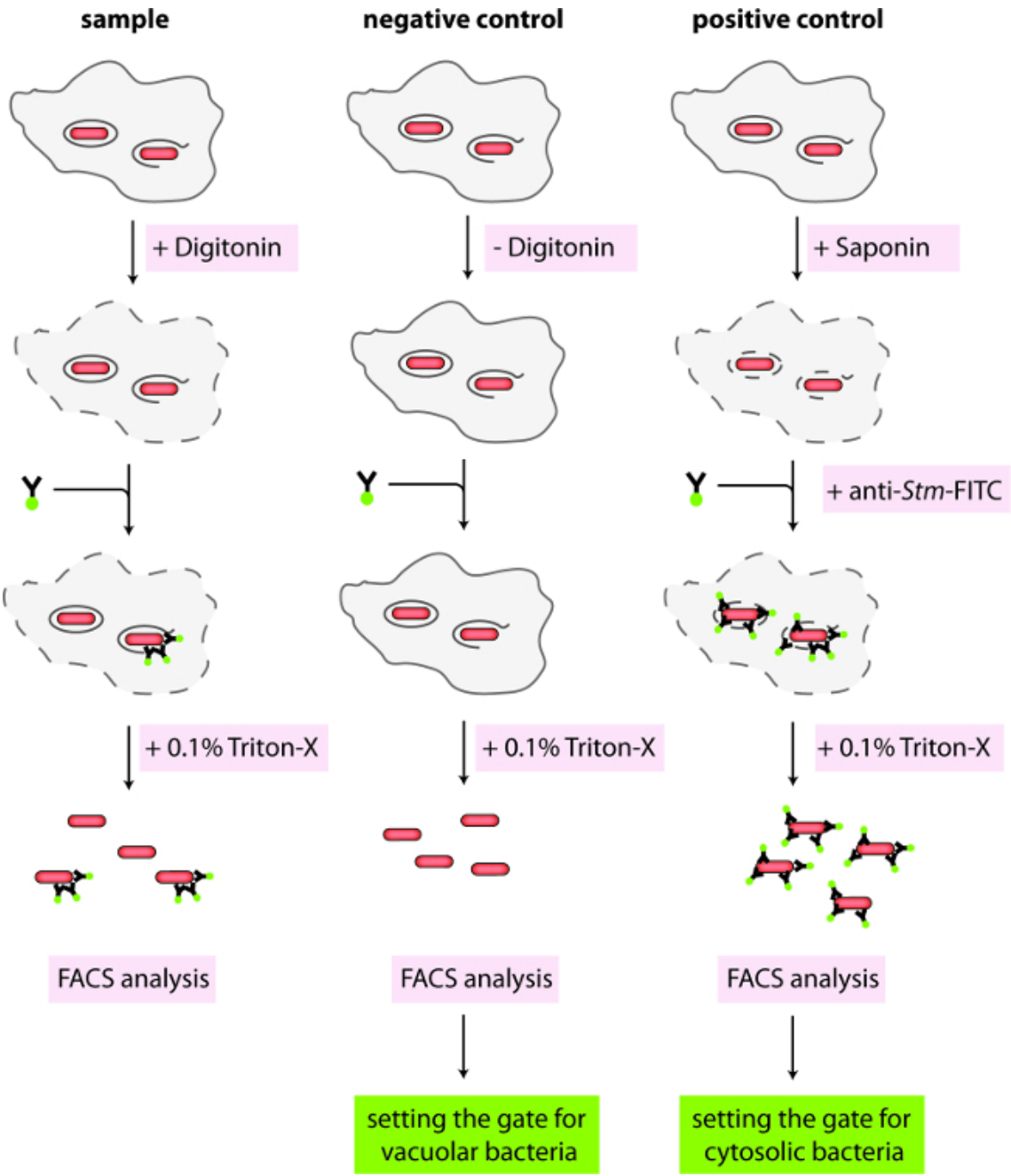

Figure 1. Schematic representation of protocol 1. Infected cells containing mCherry-positive *Salmonella* either in intact vacuoles or the cytosol are differentially permeabilized with digitonin. Cytosolic *Salmonella* are stained with anti-*Salmonella* coupled to FITC. Cells are washed and lysed with Triton X-100 for FACS analysis. Negative control cells are not permeabilized, while positive control cells are completely permeabilized before antibody staining.

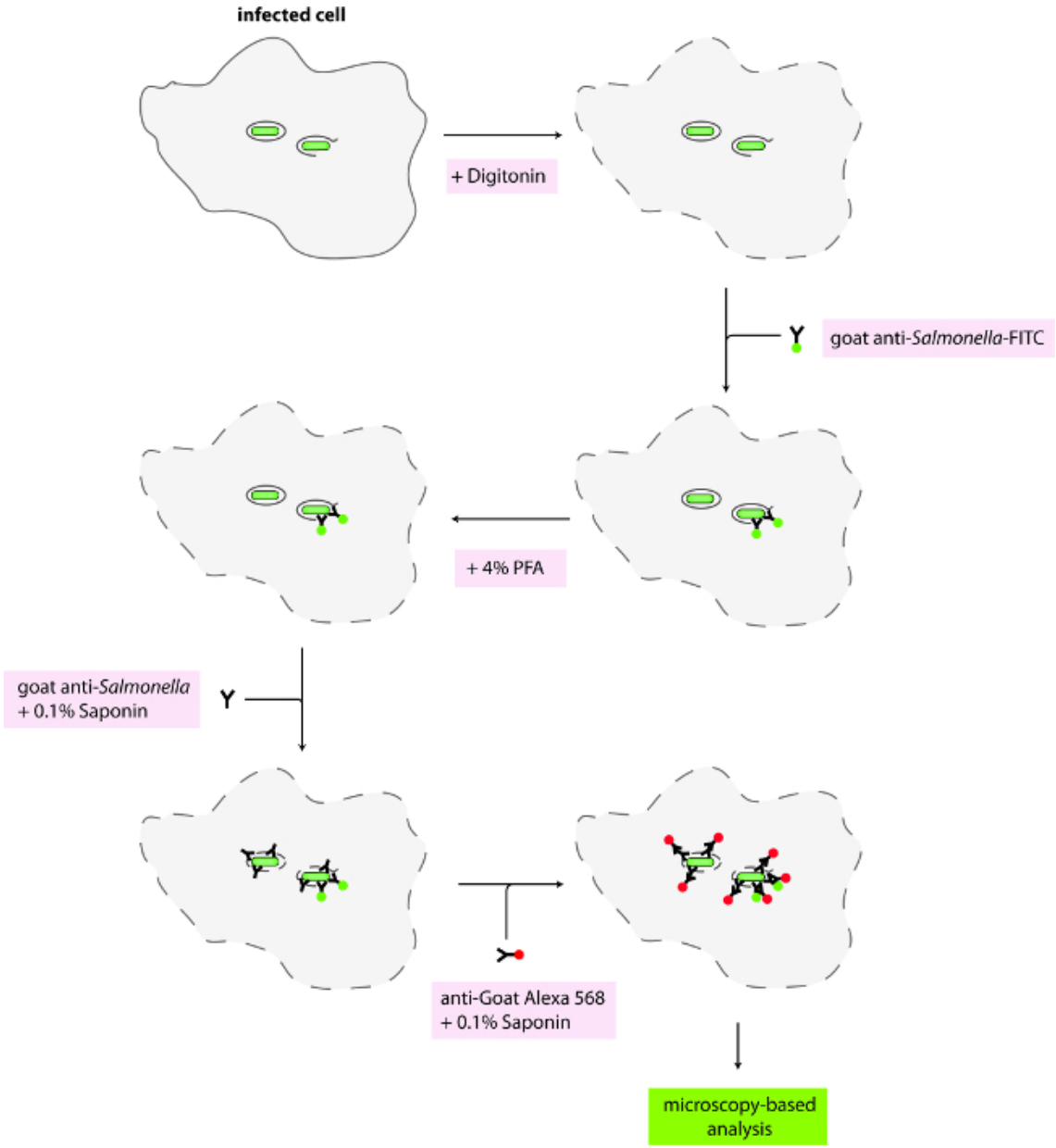

Figure 2. Schematic representation of protocol 2. Infected cells containing unlabeled *Salmonella* either in intact vacuoles or the cytosol are differentially permeabilized with digitonin. Cytosolic *Salmonella* are stained with anti-*Salmonella* coupled to FITC. Cells are washed and fixed with 4% PFA. Cells are completely permeabilzed and stained with anti-*Salmonella* antibodies, followed by staining with secondary antibodies coupled to Alexa 568. Microscopy is used to count FITC+/Alexa568+ *Salmonella* (cytosolic) and Alexa568+ *Salmonella* (vacuolar).

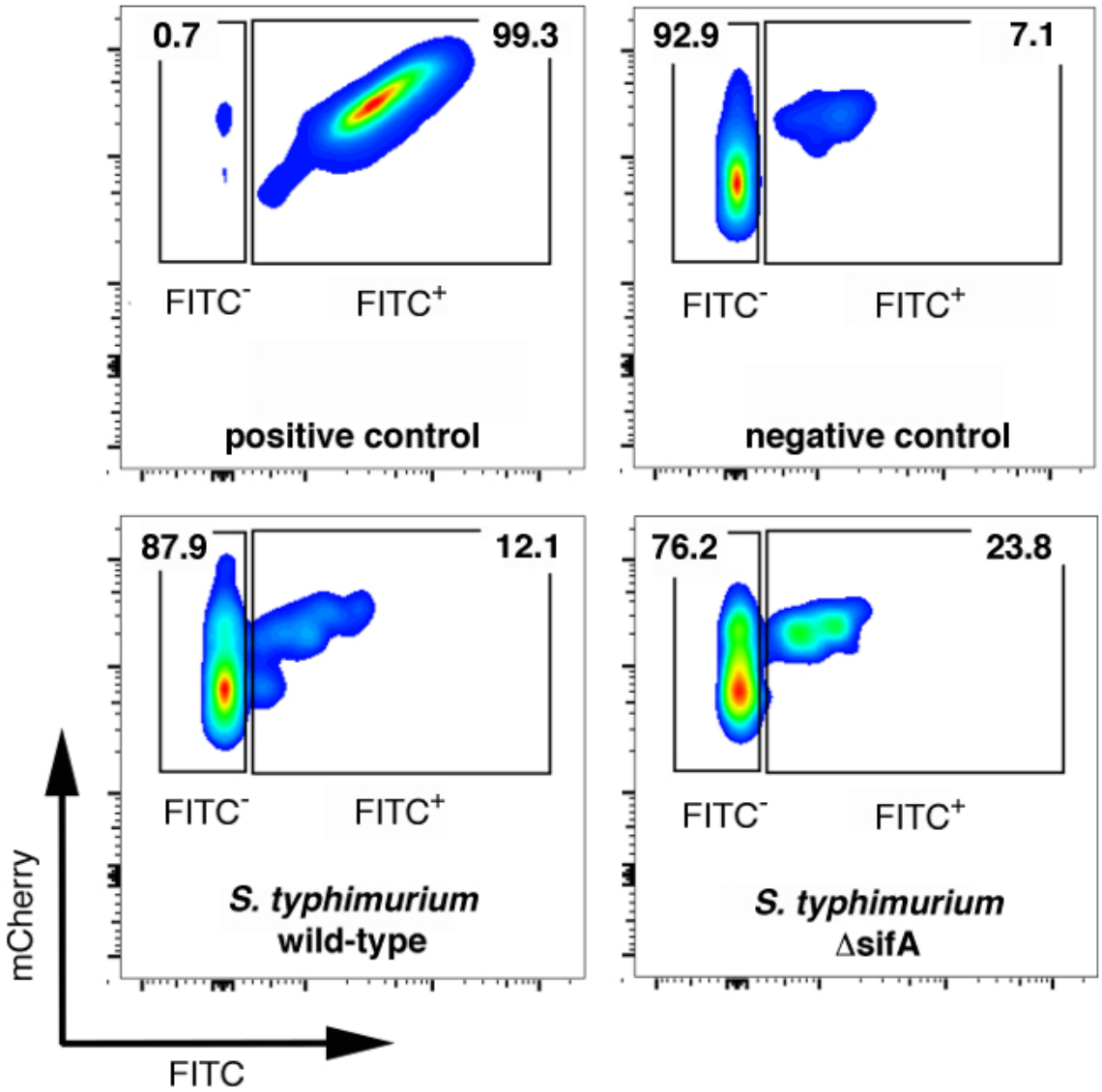

Figure 3. FACS analysis of fully permeabilized, unpermeabilized or differentially permeabilized samples infected for 6 hr with mCherry+ wild-type or ΔsifA *Salmonella* for 6 hr.

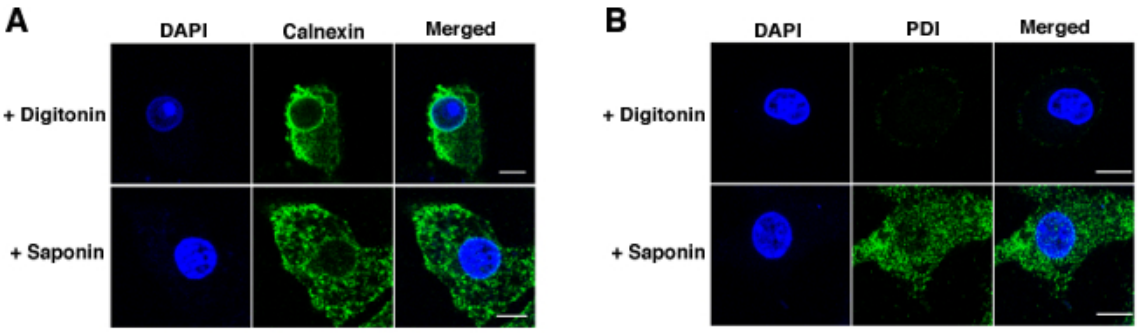

Figure 4. Anti-Calnexin (A) and anti-PDI (B) staining of uninfected BMDMs permeabilized with digitonin or saponin. Scale bars 10 µm.

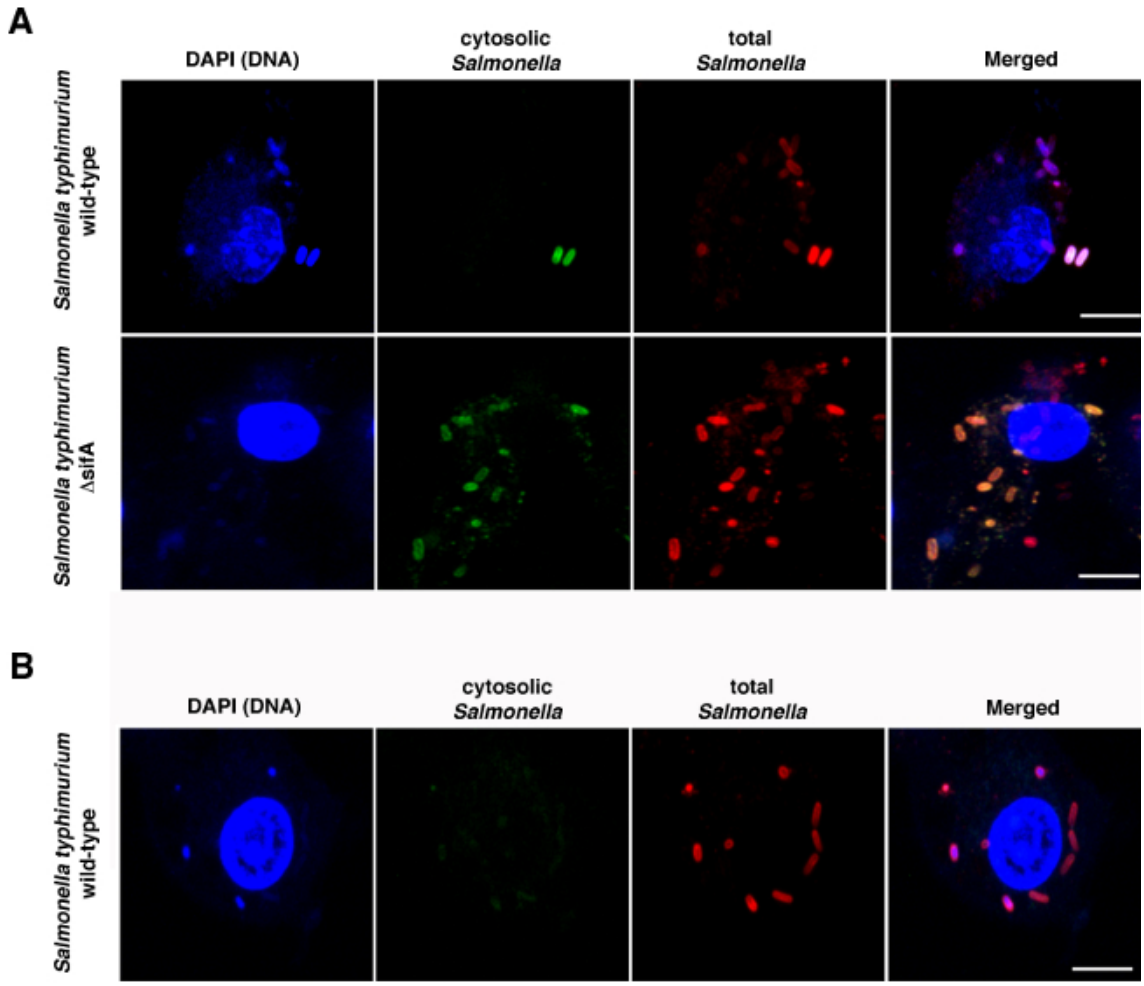

Figure 5. Representative images of a differential permeabilization assay with *Salmonella typhimurium* wild-type and the ΔsifA mutant strain at 6 hrs post-infection. Cytosolic *Salmonella* and total *Salmonella* are indicated. Cells were either digitonin permeabilized **(A)** or left unpermeabilized **(B)** before staining with anti-*Salmonella*-FITC for cytosolic *Salmonella*. After this staining step, cells were permeabilized with Saponin and total *Salmonella* were stained with anti-*Salmonella* antibodies followed by secondary antibody staining (red). Scale bars 10 µm.

Discussion

Differential permeabilization is an easy and robust method to analyze and quantify the subcellular distribution of bacterial pathogens between vacuolar compartments and the cytosol. The same assay has been used successfully with bacteria such as *Francisella novicida*[2,4] and *Shigella flexneri*[12]. However, since many intracellular pathogen alter or modify the host endomembrane structures, the robustness of the digitonin permeabilization will have to be determined on an individual basis. Fine-tuning of the assay in respect to the pathogen or cell line used might be necessary. The assay is not just restricted to infection biology, but can be also used for other cell biological application that, like cell death signaling[17,18].

The key to this assay is that the plasma membrane and intracellular membrane have a different lipid composition, in particular intracellular membranes contain less cholesterol. Digitonin can complex cholesterol and thus leads to differential permeabilization, the efficiency and selectivity of which depends on the length of digitonin treatment and the concentration of the detergent. To control for this it is important to check permeabilized cells by staining for marker proteins with defined intracellular localization, such as the cytosolic tail of calnexin or the ER luminal protein PDI.

While differential permeabilization is not new, this protocol allows in addition a fast and objective quantification of cytosolic and vacuolar bacteria based on FACS. For the FACS analysis it is important to include two controls, which are non-permeabilized and fully permeabilized cells. Based on these control samples the gates for unstained and fully stained *i.e.* vacuolar and cytosolic bacteria will be set.

A critical step of the protocol is the permeabilization and the subsequent washing steps. Freshly prepared buffer and detergent solutions have to be used. All solutions should be prepare just before use. Another critical point is the timing of the permeabilization. Here 1 min at a digitonin concentration of 50 µg/ml has proved to work well for bone-marrow derived macrophages. When using different cell lines, the working concentration might need to be determined empirically. High concentrations of digitonin or incubation times over 1 min can lead to permeabilization of internal membranes, and false positive results. Digitonin solutions are only stable for 1 - 2 hr. Preparing a fresh solution of

digitonin just before is absolutely critical. Also the capacity of digitonin to permeabilze membranes can vary from batch to batch and between different suppliers.

Therefore if many samples need to be treated it is advisable to split these up into smaller groups that can be easily and rapidly processed. Finally, all washing steps after the permeabilization need to be done very carefully since the digitonin treatment weakens cellular membranes. Addition of buffer and aspiration should be done gently at the side of the well and not in the middle of the well.

As pointed out, other techniques to analyze the subcellular distribution of bacteria between the cytosol and vacuolar compartments exist, such as the use of the b-lactamase cleavage FRET reporter CCF4-AM [19]. While the CCF4-AM method offer the advantage of measuring vacuolar rupture in real-time, it lacks the resolution at the single bacterium level and can only provide information on the single host cell level. Thus a combination of both assays is ideal to gain information on the levels of single bacteria/host cells over time. Importantly, both assays can be used not only in the context of bacterial infections, but also in the context of other cell biological studies that aim to determine the sub-cellular localization of target proteins[18].

Disclosures

We have nothing to disclose.

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