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Monitoring *Shigella flexneri* vacuolar escape by flow cytometry

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Invasive bacterial pathogens such as *Shigella flexneri* force their uptake into non-phagocytic host cells. Upon internalization, they rupture the endocytic vacuole and escape into the host cell cytoplasm. Recent studies applying fluorescence resonance energy transfer (FRET)-based methods to track host-pathogen interactions have provided insights into the process of bacterial infection at the single cell level. We have previously reported that the vacuolar escape of invasive bacteria into the host cellular cytosol can be tracked by fluorescence microscopy using a FRET CCF4/β-lactamase reporter assay. Here, we show that our vacuolar rupture assay can also be analyzed by flow cytometry constituting an important alternative to data acquisition by microscopy. Whereas analysis of our assay by fluorescence microscopy offers precise spatiotemporal resolution, flow cytometry analysis represents a high-throughput method that allows efficient and fast quantification of a large number of events and can further improve future research on vacuolar escape.

Key words: host-pathogen interaction, imaging, vacuolar rupture, *Shigella flexneri*, fluorescence resonance energy transfer, CCF4-AM, β-lactamase, reporter

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Introduction

Invasive bacterial pathogens such as Gram-negative Shigella, Salmonella and Yersinia and Gram-positive Listeria have the capacity to force their uptake into non-phagocytic host cells. This process requires rearrangements of the actin cytoskeleton at the bacterial entry site and occurs through a zipper mechanism (for Yersinia and Listeria) or macropinocytosis accompanied by membrane ruffling (for Shigella and Salmonella).[1,2] Upon uptake, bacteria are initially localized within membrane-enclosed vacuoles. Subsequently, Yersinia and Salmonella remain and replicate within membrane-bound compartments whereas Shigella and Listeria rupture the endocytic vacuole and escape into the host cell cytoplasm.[2] Vacuolar escape by *Listeria monocytogenes* has been shown to involve the pore-forming activity of the bacterial effector protein lysteriolysin O (LLO).[3,4] In the case of *Shigella flexneri*, the type 3 secretion system (T3SS) effectors IpaB and IpaC have been implicated in contact hemolysis of the vacuole.[5,6] However, the exact mechanism of vacuolar membrane rupture by Shigella remains unclear.

To monitor host-pathogen interactions at the single-cell level, new methods have been established during the last years. One of these approaches is based on fluorescence resonance energy transfer (FRET) of the cephalosporin-derived substrate called CCF4. β-lactamase-mediated cleavage of this FRET-probe was initially used as a reporter for the study of transcriptional activity and for clonal selection.[7] Recently, this assay has been adapted to measure effector translocation or internalization of invasive bacteria in vitro and in vivo by creating β-lactamase fusion proteins with bacterial effectors.[8-13] Using Shigella invasion of host cells as a model system, we have extended these approaches by establishing a novel fluorescence microscopy assay that allows the study of vacuole rupture with high spatiotemporal precision (**Fig. 1**).[14] With this assay we have shown that Shigella escapes from the vacuole in less than 10 min after host cellular contact and that the expression of the Salmonella effector proteins SifA and PipB2 inhibit the vacuolar escape of Shigella through stabilization of the vacuolar membrane. Furthermore, we have

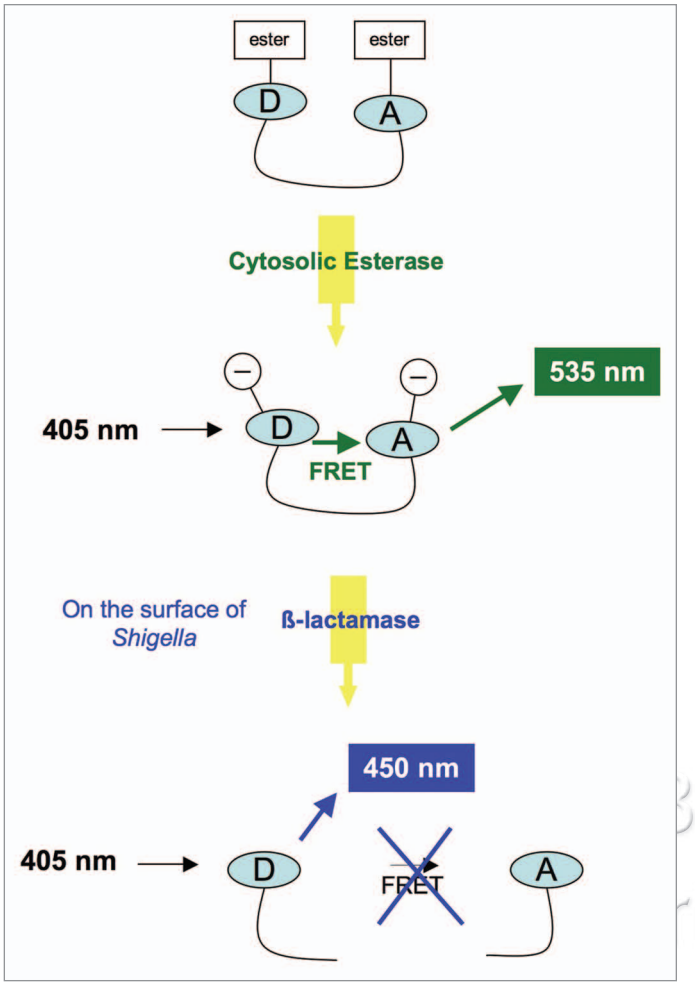

Figure 1. The principle of our CCF4/β-lactamase reporter assay. Cells (either adherent or in suspension) are loaded with the CCF4 probe. The ester moieties of the probe are cleaved and it is trapped in the cytoplasm in its anionic form. The intact probe fluoresces green (535 nm) and switches to blue (450 nm) upon cleavage, when pathogens with β-lactamase activity on their surface reach the host cytoplasm.

identified a novel host marker, galectin-3, which appears instantly in the proximity of bacteria after escape into the cytosol and "flags" cytoplasmic pathogens.[15]

Flexibility of data acquisition is essential to study host-pathogen interactions, since each experimental system harbors advantages and disadvantages that have to be balanced depending on the scientific question addressed. Previous studies on translocation of effector proteins have shown that the FRET-based β-lactamase reporter assay is compatible with analysis by flow cytometry.[10-12] In the present report we show that our vacuolar rupture assay can also be analyzed by flow cytometry, which constitutes an important alternative and complementary read-out to microscopy-based data acquisition.

Experimental Approach

Our method is based on pre-loading of host cells with the CCF4-AM probe (Invitrogen) sensitive to FRET measurements.[7] Upon cellular uptake of the probe, attached ester moieties are cleaved off and the CCF4 molecule is subsequently trapped in the host cytoplasm. Excitation of the intact CCF4 substrate at 405 nm results in FRET and emission at 535 nm (green cells). The probe is cleaved when bacteria displaying β-lactamase on their surface access the host cytosol. This leads to dequenching of the donor fluorophore and loss of FRET signal, with an emission at 450 nm (blue cells).

We have previously shown that in the case of Shigella and Salmonella, β-lactamase activity could be measured on the bacterial surface if this enzyme is expressed from plasmids that render the bacteria resistant to ampicillin.[14] Accordingly, the bacterial strains used in this study include the invasive wild-type strain of *Shigella flexneri* M90T supplemented with a plasmid for the expression of the adhesin AfaI, and the non-invasive ΔmxiD mutant strain supplemented with the same plasmid. This plasmid encodes β-lactamase via its resistance cassette. To pre-load human epithelial HeLa cells with CCF4, cells were trypsinized, transferred to a 96-well format at a concentration of 3×10^5 cells per well in 100 µl of EM buffer (120 mM NaCl, 7 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose and 25 mM Hepes at pH 7.3) with 1 µM CCF4 and incubated for 1 h at room temperature. Bacteria were grown overnight in Tryptic casein soy broth (TCSB) supplemented with 100 µg/ml ampicillin. Overnight bacterial cultures were inoculated at a 1/50 dilution in TCSB and grown to an optical density of ≈0.5 at 600 nm. Subsequently, cell infection with Shigella was carried out in suspension in DMEM medium at a multiplicity of infection (MOI) of 10 or 100 bacteria per cell. Cells were centrifuged for 10 min at 300 g and incubated for 50 min with the bacteria before adding rifampicin at a concentration of 50 µg/ml in order to inactivate extra- and intracellular bacteria. Cells were washed with PBS and transferred to flow cytometry tubes for analysis. All steps following the cell loading with CCF4 were carried out in the presence of 1 mM probenecid in order to prevent a loss of the CCF4 signal. In case cells were fixed prior flow cytometry analysis, they were incubated in 4% paraformaldehyde (PFA) for 20 min at room temperature. Data acquisition was carried out with a CyAn ADP flow cytometer (DakoCytomation). Live, single cells were gated, and the fluorescence intensities of cleaved and uncleaved CCF4 were detected with the 405 nm excitation laser and 450 nm and 535 nm emission filters. Data were analyzed with Summit 4.3 software and graph pad prism.

Results

Changes in the FRET signal only occur if the invading pathogen escapes the vacuole

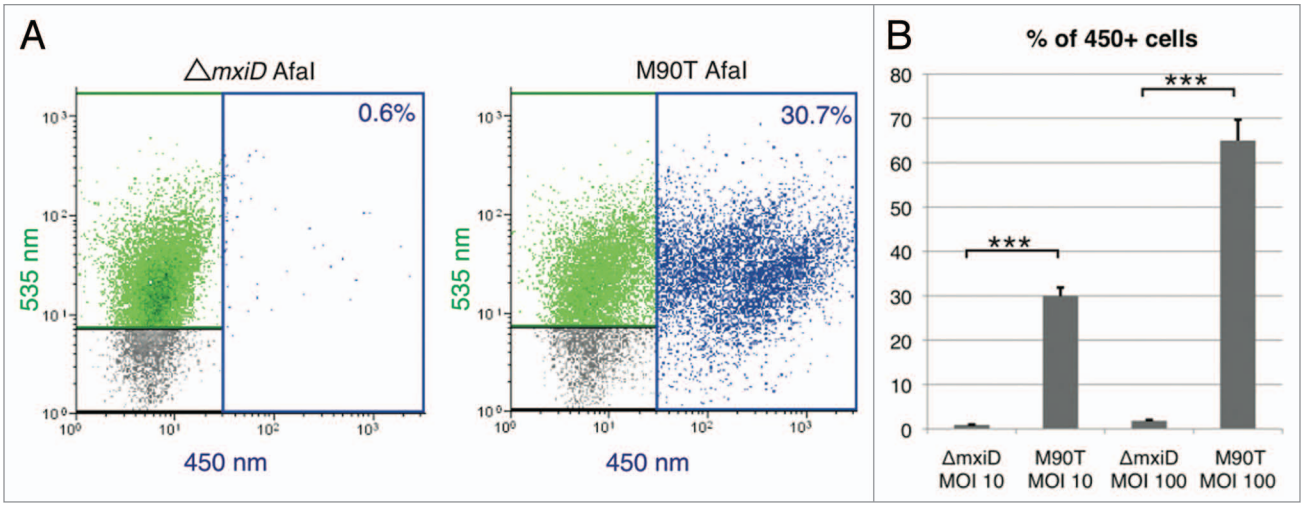

Figure 2. Flow cytometry analysis of *Shigella flexneri* vacuole escape. (A) Representative flow cytometry graphs of HeLa cells infected at a MOI of 10 with Shigella ΔmxiD AfaI (non-invasive mutant) or with Shigella M90T AfaI (invasive wild-type). Samples were acquired by flow cytometry analysis without fixation. Cleavage of the FRET probe (signal at 450 nm) is observed only for cells infected with the invasive M90T AfaI. (B) Statistical analysis of the 450+ population in Shigella ΔmxiD AfaI (ΔmxiD) and Shigella M90T AfaI (M90T) infected cells at a MOI of 10 or 100. In the sample M90T at a MOI of 10 vacuolar rupture occurred in 30.7% of cells whereas at a MOI of 100 vacuole rupture occurred in 65.0% of cells. The error bars correspond to the standard deviation and the three asterisks highlight the statistical significance ($p < 0.0001$).

and comes into contact with the host cell cytoplasm (**Fig. 1**). Through CCF4 probe excitation at 405 nm and simultaneous analysis of the emission signal at 535 nm and at 450 nm it is possible to distinguish whether the bacteria are extracellular, within the vacuole or have already escaped. We show that this change in fluorescence signal previously measured by microscopy can also be analyzed by flow cytometry (**Fig. 2**).

Analysis of CCF4 pre-loaded HeLa cells infected with invasive M90T AfaI or non-invasive ΔmxiD AfaI Shigella confirms that only in the case of infection with the invasive strain we detected an emission peak at 450 nm (**Fig. 2A**). Furthermore, the appearance of the signal at 450 nm was accompanied by a loss of signal at 535 nm. We were able to observe two distinct populations: the 535+/450- population corresponding to non-invaded cells or invaded cells where no vacuolar rupture has occurred and the 450+ population corresponding to invaded cells where vacuolar rupture has occurred and the FRET-probe has been cleaved. The 450+ population with an MOI of 10 for M90T AfaI-infected cells comprised 30.7% and for ΔmxiD AfaI-infected cells it was 0.6%, while with an MOI of 100 for the M90T AfaI sample it was 65.0% and for the ΔmxiD AfaI sample it was 1.8% (**Fig. 2B**). Altogether, this data proves that quantitative and statistically significant data can be generated by this assay. As described in previous studies, we found that the intact FRET probe was less stable inside cells than the fluorescent cleaved blue product, and we also observed that PFA fixation affected the signal of the intact FRET pair at 535 nm (data not shown).[13] M90T AfaI-infected cells (at a MOI of 10) fixed with 4% PFA displayed a diminished intensity of fluorescent emission at 535 nm as early as 10 min after fixation. Importantly, the shift to 450 nm upon vacuolar escape was still observed under these conditions indicating that the assay can also be used in conjunction with fixed samples.

Conclusion

Studies on host-pathogen interactions have often taken advantage of fluorescence microscopy or flow cytometric approaches. We have previously reported that the vacuolar escape of invasive bacteria into the cytosol of host cells can be tracked by fluorescence microscopy using a CCF4/β-lactamase reporter assay. Here we present the adaptation of our assay for flow cytometry analysis. Although the latter does not offer the precise spatiotemporal resolution of fluorescence microscopy, it constitutes a high-throughput approach, which yields information on a large number of cells and greatly increases the ability of the assay to supply statistically significant data. Future research using this approach could give important new insights into the exact mechanism by which vacuolar rupture occurs, i.e., by simultaneous analysis of multiple Shigella mutant strains and identification of bacterial effectors involved in this process. Additionally, this method can easily be coupled to other methods such as fluorescence-activated cell sorting and subsequent high-content analysis of invaded cells. Fluorescence loading and bacterial invasion of cells in suspension in a 96-well format provides further experimental flexibility, i.e., allowing the study of different cell types in parallel and miniaturization of the assay to economize the fluorescence probe. However, we have also noticed that employment of standard procedures like PFA fixation may exacerbate the readout of the applied assay. A comparative analysis of live cells and cells fixed with PFA revealed that PFA fixation leads to a diminished intensity of fluorescent emission at 535 nm (intact FRET) whereas it does not affect the population shift to 450 nm (cleaved probe). This effect could be due to a reaction of the PFA with the CCF molecule affecting the fluorescein

acceptor signal while not impairing the signal of the donor fluorophore.

Keeping the advantages and limitations of the employed assays in mind, the exploration of the read-out versatility of using either fluorescence microscopy or flow cytometry could greatly improve our understanding of vacuole rupture by invasive pathogens.

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