Video Article

Analysis of *Yersinia enterocolitica* Effector Translocation into Host Cells Using Beta-lactamase Effector Fusions

Manuel Wolters[1], Bernd Zobiak[2], Theresa Nauth[1], Martin Aepfelbacher[1]

[1]Institute for Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf

[2]UKE Microscopy Imaging Facility, University Medical Center Hamburg-Eppendorf

Correspondence to: Manuel Wolters at m.wolters@uke.de

URL: http://www.jove.com/video/53115
DOI: doi:10.3791/53115

Keywords: Infection, Issue 104, type III secretion system, translocation, Yersinia enterocolitica, beta-lactamase, effector protein, yersinia outer protein, Yop, YopE, FRET

Date Published: 10/13/2015

Citation: Wolters, M., Zobiak, B., Nauth, T., Aepfelbacher, M. Analysis of *Yersinia enterocolitica* Effector Translocation into Host Cells Using Beta-lactamase Effector Fusions. *J. Vis. Exp.* (104), e53115, doi:10.3791/53115 (2015).

Abstract

Many gram-negative bacteria including pathogenic *Yersinia spp.* employ type III secretion systems to translocate effector proteins into eukaryotic target cells. Inside the host cell the effector proteins manipulate cellular functions to the benefit of the bacteria. To better understand the control of type III secretion during host cell interaction, sensitive and accurate assays to measure translocation are required. We here describe the application of an assay based on the fusion of a *Yersinia enterocolitica* effector protein fragment (Yersinia outer protein; YopE) with TEM-1 beta-lactamase for quantitative analysis of translocation. The assay relies on cleavage of a cell permeant FRET dye (CCF4/AM) by translocated beta-lactamase fusion. After cleavage of the cephalosporin core of CCF4 by the beta-lactamase, FRET from coumarin to fluorescein is disrupted and excitation of the coumarin moiety leads to blue fluorescence emission. Different applications of this method have been described in the literature highlighting its versatility. The method allows for analysis of translocation *in vitro* and also in *in vivo, e.g.,* in a mouse model. Detection of the fluorescence signals can be performed using plate readers, FACS analysis or fluorescence microscopy. In the setup described here, *in vitro* translocation of effector fusions into HeLa cells by different Yersinia mutants is monitored by laser scanning microscopy. Recording intracellular conversion of the FRET reporter by the beta-lactamase effector fusion in real-time provides robust quantitative results. We here show exemplary data, demonstrating increased translocation by a *Y. enterocolitica* YopE mutant compared to the wild type strain.

Video Link

The video component of this article can be found at http://www.jove.com/video/53115/

Introduction

Type III secretion systems are specialized protein-export machines utilized by different genera of gram-negative bacteria to directly deliver bacterially encoded effector proteins into eukaryotic target cells. While the secretion machinery itself is highly conserved, specialized sets of effector proteins have evolved among the different bacterial species to manipulate cellular signaling pathways and facilitate specific bacterial virulence strategies[1]. In case of *Yersinia,* up to seven effector proteins, so called Yops (*Yersinia* outer proteins), are translocated upon host cell contact and act together to subvert immune cell responses such as phagocytosis and cytokine production, *i.e.,* to permit extracellular survival of the bacteria[2-4]. The process of translocation is tightly controlled at different stages[5]. It is established that primary activation of the T3SS is triggered by its contact to the target cell[6]. However, the precise mechanism of this initiation is yet to be elucidated. In *Yersinia* a second level of so called fine-tuning of translocation is achieved by up- or down-regulating activity of the cellular Rho GTP-binding proteins Rac1 or RhoA. Activation of Rac1 *e.g.* by invasin or cytotoxic necrotizing factor Y (CNF-Y) leads to increased translocation[7-9], while the GAP (GTPase activating protein) function of translocated YopE down-regulates Rac1 activity and accordingly decreases translocation by a negative feedback type of mechanism[10,11].

Valid and precise methods are the prerequisite to investigate how translocation is regulated during *Yersinia* host cell interaction. Many different systems have been used for this purpose, each with specific advantages and drawbacks. Some approaches rely on lysis of infected cells but not bacteria by different detergents followed by western blot analysis. The common drawback of these methods is that minor but inevitable bacterial lysis potentially contaminates the cell lysate with bacteria-associated effector proteins. However, treatment of cells with proteinase K to degrade extracellular effector proteins and subsequent use of digitonin for selective lysis of the eukaryotic cells were proposed to minimize this problem[12]. Importantly, these assays crucially depend on high quality anti-effector antibodies, which are mostly not commercially available. Attempts to use translational fusions of effector proteins and fluorescent proteins like GFP to monitor translocation were not successful probably due to the globular tertiary structure of the fluorescent proteins and the inability of the secretion apparatus to unfold them before secretion[13]. However, several different reporter tags like the cya (calmodulin-dependent adenylate cyclase) domain of the *Bordetella pertussis* toxin cyclolysin[14] or the Flash tag were successfully used to analyze translocation. In the former assay the enzymatic activity of cya is used to amplify the signal of the intracellular fusion protein, while the Flash tag, a very short tetracysteine (4Cys) motif tag, allows for labeling with the biarsenical dye Flash without disturbing the process of secretion[15].

Copyright © 2015 Journal of Visualized Experiments

The approach applied here was reported for the first time by Charpentier *et al.* and is based on the intracellular conversion of the Förster resonance energy transfer (FRET) dye CCF4 by translocated effector TEM-1 beta-lactamase fusions[16] (**Figure 1A**). CCF4/AM is a cell-permeant compound in which a coumarin derivate (donor) and a fluorescein moiety (acceptor) are linked by a cephalosporin core. Upon passive entry into the eukaryotic cell, the non-fluorescent esterified CCF4/AM compound is processed by cellular esterases to the charged and fluorescent CCF4 and thereby trapped within the cell. Excitation of the coumarin moiety at 405 nm results in FRET to the fluorescein moiety, which emits a green fluorescence signal at 530 nm. After cleavage of the cephalosporin core by the beta-lactamase FRET is disrupted and excitation of the coumarin moiety leads to blue fluorescence emission at460 nm. Different applications of this method have been described in the literature highlighting its versatility. The method allows for analysis of translocation *in vitro* and also in *in vivo, e.g.,* the technique was used in a mouse infection model to identify the leukocyte populations targeted for translocation *in vivo*[17-19]. Readout of signals can be conducted using plate readers, FACS analysis or fluorescence microscopy. Of note, the method also provides the possibility to monitor translocation in real-time by live-cell microscopy during the infection process[20,21]. Here laser scanning fluorescence microscopy was applied for readout of fluorescence signals as it provides highest sensitivity and accuracy. In particular, the capability to adjust the emission window with nanometer precision in combination with high-sensitive detectors facilitates optimized fluorescence detection and minimized cross-talk. In addition this microscopy setup can be adapted for real-time monitoring of translocation and potentially permits for simultaneous analysis of host-pathogen interaction at the cellular level.

In this study translocation rates of a *Y. enterocolitica* wild type strain and a YopE deletion mutant exhibiting a hypertranslocator phenotype[10,11] were exemplarily analyzed.

Protocol

1. Peak Emission and Cross-talk Determination (See Also Figure 2)

1. In order to determine the emission peaks and amount of cross-talk between the donor (coumarin derivate V450) and acceptor (fluorescein) dyes, perform spectral scans of cells labeled with both individual fluorophores at 405 nm excitation.
2. Determine a 10 nm bandwidth within the peak of each dye that will be used for the donor and acceptor channels (here 455-465 nm and 525-535 nm). Plot the normalized intensity over the wavelength and calculate the average percentage of cross-talk of the donor dye in the acceptor channel and *vice versa* (**Figure 2**).

2. Preparation of *Y. enterocolitica* Strains for Cell Culture Infection

1. Use the following strains: WA-314 pMK-ova (WA-314 transformed with the plasmids pMK-bla[17] and pMK-ova[17], respectively) and WA-314ΔYopE pMK-bla (WA-314ΔYopE transformed with the plasmid pMK-bla[17])
2. At least two days before infection streak *Y. enterocolitica* strains WA-314 pMK-ova, WA-314 pMK-bla and WA-314ΔE pMK-bla from cryo stocks on LB-agar plates containing the required antibiotics (kanamycin for WA-314 pMK-bla and WA-314 pMK-ova; kanamycin and tetracycline for WA-314ΔE pMK-bla) and grow O/N at 27 °C. Afterwards keep the plates for up to one week at 4 °C.
3. One day before infection inoculate 3 ml of LB-medium containing the required antibiotics with one colony of the respective strains and incubate O/N at 27 °C in a shaker at 180 rpm.
4. On the day of infection inoculate 2 ml of the O/N culture into 40 ml of fresh LB-medium without antibiotics and incubate for 90 min at 37 °C in a shaker at 180 rpm to induce expression of the type III secretion system.
5. Transfer the cultures into a suitable tube and keep the cultures on ice or at 4 °C from now on. Centrifuge the cultures for 10 min at 5,000 x g and 4 °C.
6. Resuspend the bacterial pellet in 2-3 ml of ice cold PBS. Dilute the bacterial suspension to a concentration of 8 x 10^8 cfu/ml by using a spectrophotometer. Determine the corresponding optical density individually. Keep this suspension on ice until infecting the HeLa cells.

3. Plating HeLa Cells

1. One day before infection seed 1.5 x 10^4 cells HeLa cells in 200 µl DMEM containing 10% heat inactivated fetal calf serum (FCS) in wells of a 96 well plate and cultivate in a 5% CO_2 incubator at 37 °C. Seed three wells for each strain (three different incubation times).

4. Infection of HeLa Cells and Loading with CCF4

1. Infect HeLa cells by adding 3.5 µl of the bacterial suspension to the medium and mix by gently pipetting the medium twice. Allow the bacteria to sediment onto the cells at a MOI (moiety of infection) of about 100 bacteria per cell. For a time course start infections at -90 min, -60 min and -30 min and incubate at 37 °C in a 5% CO_2 incubator until 0 min to achieve a common end point.
2. When incubation is completed, carefully aspirate the medium without removing HeLa cells and add 50 µl of PBS containing 20 µg/ml chloramphenicol to stop expression of effectors and 2.5 mM probenecid to reduce export of CCF4.
3. At this point transfer the 96-well plate into the microscope stage and define suitable spots for later analysis in each well. Finish this step within 10 min (for details of acquisition see section 5.1).
4. Per well, add 70 µl of CCF4/AM loading solution (0.24 µl solution A, 1.2 µl solution B, 18.56 µl solution C (solution A, B, and C provided within CCF4/AM loading kit) and 50 µl PBS (containing 20 µg/ml chloramphenicol and 2.5 mM probenecid) using a multi pipette and incubate for 5 min at RT. From now on work without interruptions.
5. Aspirate the loading solution and add 100 µl of PBS containing 20 µg/ml chloramphenicol and 2.5 mM probenecid using a multi pipette. Then rapidly transfer the 96 well plate into the microscope stage and start acquisition within 10 min from aspiration of the loading solution.

Copyright © 2015 Journal of Visualized Experiments

5. Laser Scanning Microscopy and Analysis of Data

1. Image acquisition
    1. Set the imaging conditions in a way to maximize the total photon count and minimize phototoxicity, photobleaching and cross-excitation.
        1. In a modern confocal laser scanning microscope, use a 20X high numerical aperture immersion objective, open the detector pinhole to 2.5 Airy units (*i.e.,* wide optical sectioning), choose high sensitivity GaAsP-detectors with simultaneously active donor and acceptor channels, 8 bit depth, ~750 nm pixel size, 3-fold frame averaging and excite with 10% of a 405 nm diode laser.
        2. To ensure correct quantification set the detector gain of both channels to the same value and to avoid any saturated pixels. Note: Here, the gains were set to 150%.
    2. Ensure continuous immersion by spreading immersion medium to the bottom of the wells, for example with a 1 ml syringe, and avoid trapping of any air bubbles.
    3. Activate the transmitted light and find a representative field of view in every well. Use a correctly calibrated automatic stage and mark the position in the software.
    4. After removal of the plate for staining (see section 4.3), reinsert the plate and add a weight on top to avoid focal drift. Review the saved positions with the laser scanning mode and adjust the focus and lateral position properly.
    5. Set the time lapse to 2 min, the duration to 2 hr and start acquisition.
2. Image quantification (examples will be given for Bitplane Software such as Imaris)
    1. Import the dataset into software that allows arithmetic calculations of pixel matrices. Do this by dragging and dropping the source file containing both the donor and the acceptor channel on the software icon.
    2. Subtract the background in both channels using the same offset. To do this, click *Menu* and then click Image>Processing>Baseline Subtraction. Select each channel and enter the baseline value.
    3. Correct the bleed-through of the donor channel (Ch1) into the acceptor channel (Ch2) with the pre-determined correction factor f (see 1.1), creating a new channel:
    Ch2' = Ch2 – Ch1 * f
    NOTE: A bleed-through of the acceptor into the donor channel was not detectable over the level of noise.
        1. Click Menu>Image Processing>Channel Arithmetics and enter *abs*(*ch2-(ch1*0.33))*. Afterwards delete Channel 2 by clicking Menu>Edit>Delete Channels. Ensure that the bleed-through corrected acceptor channel remains as the new Channel 2. Optional: Change the channel color from gray to the original color by going to Menu>Edit>Image Properties. Select Channel 4>Mapped Color>Base Color and change it to *e.g.,* green.
    4. Create a new channel with the following operation to determine the relative FRET coefficient:
    Ch3 = Ch2' / (Ch1 + Ch2')
        1. Go to Menu>Image Processing>Channel Arithmetics and enter *ch2./(ch1+ch2)*
    5. For proper visualization of the FRET channel in an 8 bit image, create a fourth channel:
    Ch4 = 255 * Ch3
        1. Go to Menu>Image Processing>Channel Arithmetics and enter *255*ch3.* Change the channel color by going to Menu>Edit>Image Properties. Select Channel 4>Mapped Color>Color Table File>Jet.
    6. Apply a 3x3 median filter to channels Ch3 and Ch4 that will reduce the noise in the images. To do this, click to Menu>Image Processing>Smoothing>Median Filter. Select both channels and apply a filter size "3x3x1".
    7. For quantification of cellular signals, detect the cells in Ch4 by properly setting the offset and filter the structures by size to exclude too small structures.
        1. Select the *Surpass View* and click on the icon "*Add new Surfaces*". Define the detection algorithm parameters in the Surface window. For faster processing, activate the two checkboxes "Segment only a Region of Interest" and "Process entire Image finally". Define the region of interest by editing its dimensions.
        2. Select Channel 4 as the source channel and set the threshold by Thresholding>Background Subtraction (Local Contrast) and set diameter to 10 µm. Set the minimum intensity threshold manually to 0 and the maximum threshold to the maximum intensity. Filter the structures by area and set the minimum value to *e.g.,* 187 µm². Finish the surface detection.
    8. Extract the mean values for donor fluorescence intensity (Ch1) and relative FRET coefficient (Ch3) and their standard deviations in the cellular entity. To do this, go to the Surface properties. Change the appearance of the structures by selecting Surfaces Style/Quality>Surface. Select all structures by clicking on the filters tab. Unify the detected structures by going to Process Selection>Unify. Export the surface statistics to MS Excel by clicking in the statistics tab>Export All Statistics to File.
    9. Plot these values over time. Identify the 10 min interval of maximum slope of donor channel fluorescence increase for each condition as a reasonable parameter to represent the amount of translocated effector. Calculate the slope as m = Δ donor fluorescence intensity / Δ time (min).

Representative Results

To demonstrate the capability of the described method to quantitatively analyze effector translocation into target cells, two *Yersinia* strains with different translocation kinetics were studied: *Y. enterocolitica* wild type strain WA-314 (serogroup O8, harboring the virulence plasmid pYVO8[22]) and its derivative WA-314ΔYopE (WA-314 harboring pYVO8ΔYopE[23]). Earlier work showed that *Y. enterocolitica* mutants lacking functional YopE exhibit a significantly higher Yop translocation rate[10]. Both strains were transformed with vectors encoding for fusions of the n-terminal

secretion signal of YopE and TEM-1 beta-lactamase (pMK-bla)[17]. WA-314 was additionally transformed with a vector encoding for a respective YopE ovalbumin fusion (pMK-ova[17]) to serve as a negative control. Incubation times of 30, 60 and 90 min were applied in order to further assess the method`s effective range and suitability for quantification. Two different readouts were used for data analysis: donor fluorescence (Ch1) and relative FRET coefficients (Ch3 = Ch2' / (Ch1 + Ch2')) (**Figure 1B** and **1C**).

For the negative control strain WA-314-pMK-ova plotting of donor channel intensities (Ch1) over time shows no rise of fluorescence emission irrespective of the incubation time (**Figure 1B**). In contrast, in WA-314-pMK-bla infected cells an increase of fluorescence intensity over time was detected indicating the presence of YopE beta-lactamase fusion in the target cell cytoplasm. As expected the maximum slope of fluorescence intensity is positively correlated with the length of infection (30 min of infection: 0.10/min, 60 min infection: 0.33/min, 90 min infection: 0.76/min) indicating that different concentrations of translocated beta-lactamase can be distinguished. In accordance with previous studies WA-314ΔYopE-pMK-bla infected cells exhibit more rapid increase of fluorescence intensity compared to the wild type strain (30 min of infection: 1.28/min, 60 min infection: 1.53/min, 90 min infection: 1.41/min). Interestingly, expanding infection to 90 min did not further accelerate the rise of donor fluorescence compared to 60 min of infection (**Figure 1B**). This finding can presumably be explained by the progressive cytotoxic effect exerted by WA-314ΔYopE-pMK-bla, which could either inhibit efficient translocation beyond 60 min of infection or lead to loss of fluorescence dyes due to disruption of plasma membrane integrity. The results provided by using the relative FRET coefficient (Ch3) for analysis are in good accordance with the results obtained by the donor channel alone. However, in some conditions (WA-314ΔYopE-pMK-bla, 60 and 90 min infection) representing very large amounts of translocated beta-lactamase fusion, obviously the CCF4 substrate was instantly processed before imaging could be started. These conditions would not allow for calculating a reasonable maximum negative slope because the relevant part of the curve is missed in this data representation (**Figure 1C**). Images of the relative FRET coefficient channel provide the possibility to compare differences between individual cells (**Figure 1D**).

Copyright © 2015 Journal of Visualized Experiments

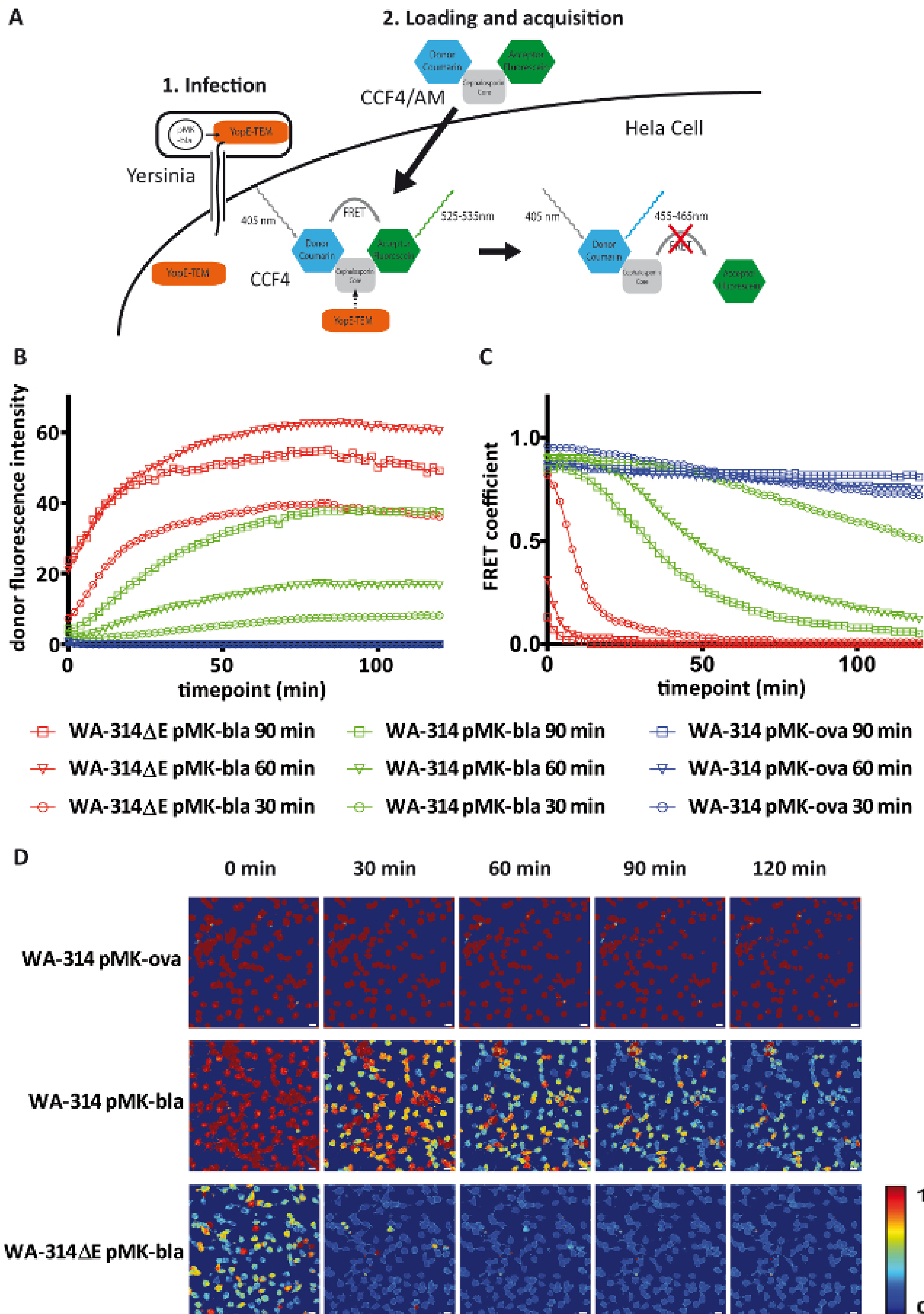

Figure 1. Quantitative analysis of effector translocation by TEM-1 YopE fusions in *Y. enterocolitica*. (A) Hydrolysis of CCF4 substrate by translocated TEM-1 beta-lactamase was monitored by laser scanning microscopy. Excitation at 405 nm resulted in green fluorescence emission (530 nm) of the intact substrate and in blue fluorescence emission (460 nm) of the cleaved hydrolysis product (coumarin). **(B, C)** Microscopy data were quantitatively analyzed by plotting the donor channel intensity (Ch1) or calculation and plotting of the relative FRET coefficients (Ch3 = Ch2' / (Ch1 + Ch2')). Data are representative of three independent experiments. **(D)** Depicted micrographs (FRET channel) were taken from representative movies at the indicated time points. WA-314ΔE-pMK-bla infected cells show more rapid decrease of the relative FRET coefficients compared to the wild type WA-314-pMK-bla (60 min infection). Scale bar, 20 µm. Please click here to view a larger version of this figure.

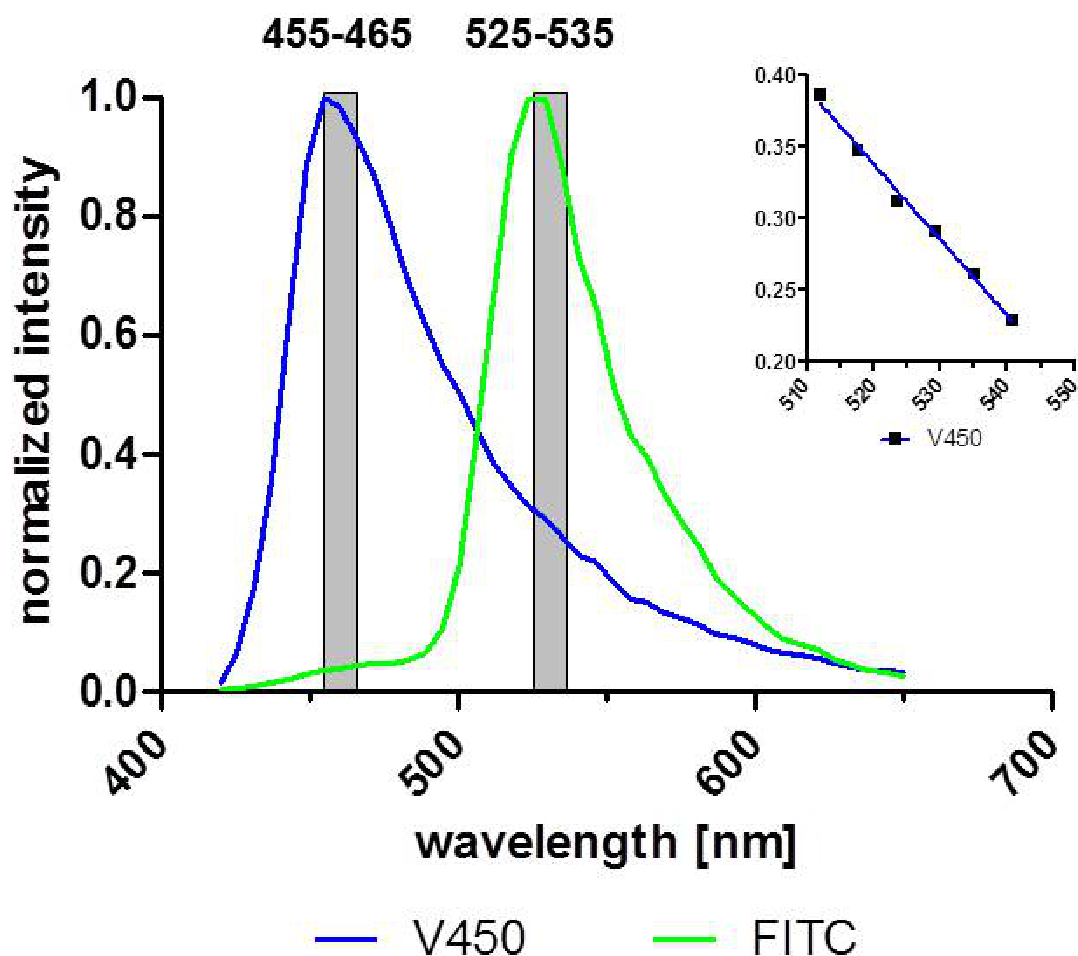

Figure 2. Cross-talk determination of the fluorophores V450 and FITC. The spectra of both individual fluorophores were measured with the confocal microscope. Bleed-through of the donor V450 into the acceptor channel within the 525-535 nm detector range was calculated from a linear regression (insert). The mean percentage of crosstalk calculated from two measurements equals 0.33. Bleed-through of the acceptor FITC into the donor channel (455-465 nm) was not detectable over the level of noise. Please click here to view a larger version of this figure.

Discussion

We here successfully applied a TEM-1 beta-lactamase reporter based assay for quantitative analysis of effector translocation by *Y. enterocolitica*. Many different variations of this sensitive, specific and relatively straightforward technique have been described in the literature. In this study laser scanning microscopy was conducted for most sensitive and precise detection of fluorescence signals. Specifically the correction for cross-talk between the donor and acceptor dyes and the individually adjustable detection bandwidths allow for superior accuracy of measurement compared to other variations of the method. The results clearly show that the method is capable of quantitatively analyzing translocation. Different incubation times representing different concentrations of intracellular beta-lactamase were positively correlated with the slope of donor fluorescence intensity. Also a significantly higher translocation rate of the WA-314ΔYopE compared to the wild type strain WA-314 could be demonstrated.

Two alternative options for data analysis were applied: Donor channel intensities and relative FRET coefficients. The advantage of using the donor channel is that it directly represents the accumulation of a CCF-4 hydrolysis product (coumarin). This approach is possible in a microscopic setup, because other than in a plate reader correction for differences in cell densities by using a ratio of donor/acceptor is not necessary. However, export of the CCF4 dye or its hydrolysis products is still a possible confounder in this approach. This disadvantage can be overcome by calculating the relative FRET coefficient.

In the described setup experiments were conducted in 96 well plates. This format allows for testing of multiple conditions in a single experiment and helps to save expensive chemicals (specifically CCF4/AM loading kit). In the described workflow it is critical to start image acquisition at a defined time point shortly after addition of the CCF4/AM loading solution because hydrolysis of CCF4 by the translocated beta-lactamase starts immediately. In case of processing to many conditions at once, adjustment of focus and lateral positions (step 5.1.4) may take too long and hamper a timely start of image acquisition. Therefore, the maximum number of parallel samples needs to be determined individually.

One of the main problems encountered using this method was the considerable export of CCF4 substrate by the HeLa cells at 37 °C. This phenomenon could not be sufficiently counteracted by treating the cells with probenecid. We therefore decided, instead of monitoring translocation during the ongoing infection as described previously, to first complete infection and afterwards load the cells with the CCF4/AM dye. With this approach it was possible to monitor the processing of the CCF4 substrate at RT. Under these conditions reduced export of CCF4

Copyright © 2015 Journal of Visualized Experiments

was observed. The amount of export of CCF4 substrate seems to be cell line specific; the setup described here allows for reliable analysis of translocation also in cell lines, which strongly export CCF4 at 37 °C.

Export of CCF4 is not a problem in some other cell lines allowing to adopt the microscopy setup for monitoring the processes of infection and translocation simultaneously in real-time. This provides the possibility to analyze translocation into individual cells and could be used to correlate translocation with specific events of host cell interaction like bacterial adhesion or formation of micro-colonies[20]. Therefore, this assay is a valuable tool to further elucidate the mechanisms of how translocation is regulated during the interplay of bacteria and host cells.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Dr. Antonio Virgilio Failla for providing the FRET acquisition quantification algorithm and Erwin Bohn for providing the pMK-bla and pMK-ova constructs.

References

1. Galan, J. E., Lara-Tejero, M., Marlovits, T. C., & Wagner, S. Bacterial Type III Secretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells. *Annu Rev Microbiol.* **68** 415-438, doi:10.1146/annurev-micro-092412-155725, (2014).
2. Aepfelbacher, M., Trasak, C., & Ruckdeschel, K. Effector functions of pathogenic Yersinia species. *Thromb Haemost.* **98** (3), 521-529, doi:10.1160/TH07-03-0173, (2007).
3. Heesemann, J., Sing, A., & Trulzsch, K. Yersinia's stratagem: targeting innate and adaptive immune defense. *Curr Opin Microbiol.* **9** (1), 55-61, doi:10.1016/j.mib.2005.10.018, (2006).
4. Viboud, G. I., & Bliska, J. B. Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu Rev Microbiol.* **59** 69-89, doi:10.1146/annurev.micro.59.030804.121320, (2005).
5. Dewoody, R. S., Merritt, P. M., & Marketon, M. M. Regulation of the Yersinia type III secretion system: traffic control. *Front Cell Infect Microbiol.* **3** 4, doi:10.3389/fcimb.2013.00004, (2013).
6. Pettersson, J. *et al.* Modulation of virulence factor expression by pathogen target cell contact. *Science.* **273** (5279), 1231-1233, doi:10.1126/science.273.5279.1231, (1996).
7. Schweer, J. *et al.* The cytotoxic necrotizing factor of Yersinia pseudotuberculosis (CNFY) enhances inflammation and Yop delivery during infection by activation of Rho GTPases. *PLoS Pathog.* **9** (11), e1003746, doi:10.1371/journal.ppat.1003746, (2013).
8. Wolters, M. *et al.* Cytotoxic necrotizing factor-Y boosts Yersinia effector translocation by activating Rac protein. *J Biol Chem.* **288** (32), 23543-23553, doi:10.1074/jbc.M112.448662, (2013).
9. Mejia, E., Bliska, J. B., & Viboud, G. I. Yersinia controls type III effector delivery into host cells by modulating Rho activity. *PLoS Pathog.* **4** (1), e3, doi:10.1371/journal.ppat.0040003, (2008).
10. Aili, M. *et al.* Regulation of Yersinia Yop-effector delivery by translocated YopE. *Int J Med Microbiol.* **298** (3-4), 183-192, doi:10.1016/j.ijmm.2007.04.007, (2008).
11. Gaus, K. *et al.* Destabilization of YopE by the ubiquitin-proteasome pathway fine-tunes Yop delivery into host cells and facilitates systemic spread of Yersinia enterocolitica in host lymphoid tissue. *Infect Immun.* **79** (3), 1166-1175, doi:10.1128/IAI.00694-10, (2011).
12. Nordfelth, R., & Wolf-Watz, H. YopB of Yersinia enterocolitica is essential for YopE translocation. *Infect Immun.* **69** (5), 3516-3518, doi:10.1128/IAI.69.5.3516-3518.2001, (2001).
13. Radics, J., Konigsmaier, L., & Marlovits, T. C. Structure of a pathogenic type 3 secretion system in action. *Nat Struct Mol Biol.* **21** (1), 82-87, doi:10.1038/nsmb.2722, (2014).
14. Sory, M. P., & Cornelis, G. R. Translocation of a hybrid YopE-adenylate cyclase from Yersinia enterocolitica into HeLa cells. *Mol Microbiol.* **14** (3), 583-594, doi:10.1111/j.1365-2958.1994.tb02191.x, (1994).
15. Schlumberger, M. C. *et al.* Real-time imaging of type III secretion: Salmonella SipA injection into host cells. *Proc Natl Acad Sci U S A.* **102** (35), 12548-12553, doi:10.1073/pnas.0503407102, (2005).
16. Charpentier, X., & Oswald, E. Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic Escherichia coli effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. *J Bacteriol.* **186** (16), 5486-5495, doi:10.1128/JB.186.16.5486-5495.2004, (2004).
17. Koberle, M. *et al.* Yersinia enterocolitica targets cells of the innate and adaptive immune system by injection of Yops in a mouse infection model. *PLoS Pathog.* **5** (8), e1000551, doi:10.1371/journal.ppat.1000551, (2009).
18. Geddes, K., Cruz, F., & Heffron, F. Analysis of cells targeted by Salmonella type III secretion *in vivo*. *PLoS Pathog.* **3** (12), e196, doi:10.1371/journal.ppat.0030196, (2007).
19. Marketon, M. M., DePaolo, R. W., DeBord, K. L., Jabri, B., & Schneewind, O. Plague bacteria target immune cells during infection. *Science.* **309** (5741), 1739-1741, doi:10.1126/science.1114580, (2005).
20. Mills, E., Baruch, K., Aviv, G., Nitzan, M., & Rosenshine, I. Dynamics of the type III secretion system activity of enteropathogenic Escherichia coli. *MBio.* **4** (4), doi:10.1128/mBio.00303-13, (2013).
21. Mills, E., Baruch, K., Charpentier, X., Kobi, S., & Rosenshine, I. Real-time analysis of effector translocation by the type III secretion system of enteropathogenic Escherichia coli. *Cell Host Microbe.* **3** (2), 104-113, doi:10.1016/j.chom.2007.11.007, (2008).
22. Heesemann, J., & Laufs, R. Construction of a mobilizable Yersinia enterocolitica virulence plasmid. *J Bacteriol.* **155** (2), 761-767 (1983).
23. Zumbihl, R. *et al.* The cytotoxin YopT of Yersinia enterocolitica induces modification and cellular redistribution of the small GTP-binding protein RhoA. *J Biol Chem.* **274** (41), 29289-29293, doi:10.1074/jbc.274.41.29289, (1999).

Copyright © 2015 Journal of Visualized Experiments