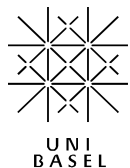


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Microscopy-based assays for high-throughput screening of host factors involved in Brucella infection of HeLa cells --Manuscript Draft--

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Abstract:	<p>Brucella species are facultative intracellular pathogens that infect animals as their natural hosts. Transmission to humans is most commonly caused by direct contact with infected animals or by ingestion of contaminated food and can lead to severe chronic infections.</p> <p>Brucella can invade professional and non-professional phagocytic cells and replicates within endoplasmic reticulum (ER)-derived vacuoles. The host factors required for Brucella entry into host cells, avoidance of lysosomal degradation, and replication in the ER-like compartment remain largely unknown. Here we describe two assays to identify host factors involved in Brucella entry and replication in HeLa cells. The protocols describe the use of RNA interference, while alternative screening methods could be applied. The assays are based on the detection of fluorescently labeled bacteria in fluorescently labeled host cells using automated wide-field microscopy. The</p>

	<p>fluorescent images are analyzed using a standardized image analysis pipeline in CellProfiler which allows single cell-based infection scoring.</p> <p>In the endpoint assay, intracellular replication is measured two days after infection. This allows bacteria to traffic to their replicative niche where proliferation is initiated around 12 h after bacterial entry. Brucella which have successfully established an intracellular niche will thus have strongly proliferated inside host cells. Since intracellular bacteria will greatly outnumber individual extracellular or intracellular non-replicative bacteria, a strain constitutively expressing GFP can be used. The strong GFP signal is then used to identify infected cells.</p> <p>In contrast, for the entry assay it is essential to differentiate between intracellular and extracellular bacteria. Here, a strain encoding for a tetracycline-inducible GFP is used. Induction of GFP with simultaneous inactivation of extracellular bacteria by gentamicin enables the differentiation between intracellular and extracellular bacteria based on the GFP signal, with only intracellular bacteria being able to express GFP. This allows the robust detection of single intracellular bacteria before intracellular proliferation is initiated.</p>
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The Editor
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Manuscript Submission to the *Journal of Visualized Experiments*

October 23rd, 2015

Dear Editor

We are submitting the manuscript entitled “High-throughput microscopy-based RNA interference assays to study host factors involved in *Brucella* infection of HeLa cells” for your consideration as article in the *Journal of Visualized Experiments*.

In this study we describe two fluorescence microscopy-based phenotypic assays in high-throughput format that allow screening of host factors involved in epithelial cells infection by *Brucella* using RNA interference. These assays facilitating the identification of host factors involved in distinct steps of the intracellular lifestyle of this important zoonotic bacterial pathogen can be adapted by any laboratory studying *Brucella* infection that has access to fluorescence microscopy. JoVE’s unique multimedia format is ideally suited to instruct interested scientists in the involved laboratory procedures and image analysis routines.

Author contributions are as follows:

- Full list of authors: Alain Casanova (AC), Shyan Huey Low (SHL), Mario Emmenlauer (ME), Raquel Conde-Alvarez (RC), Suzana P. Salcedo (SS), Jean-Piere Gorvel (JPG) and Christoph Dehio (CD)
- AC, SHL, RC, SS: developed and performed experiments
- AC, SHL, RC, SS, JPG, CD: conceived experiments and analysis
- ME: developed image analysis pipeline
- AC, SHL, ME, CD: wrote manuscript

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Thank you for your consideration of our communication. Please don't hesitate to contact me if there are any further questions. Otherwise we are looking forward to hearing from you in due course regarding your editorial decision.

With best regards,

A handwritten signature in purple ink, consisting of a stylized 'C' followed by a horizontal line and a small loop.

Prof. Christoph Dehio (Ph.D.)

TITLE:

Microscopy-based assays for high-throughput screening of host factors involved in *Brucella* infection of HeLa cells

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KEYWORDS:

siRNA (small interfering RNA), RNAi (RNA interference), high-content/high-throughput screening assay, automated image analysis, fluorescence microscopy, infection biology, cell biology, *Brucella abortus*, intracellular pathogen, HeLa cells, cell invasion

SHORT ABSTRACT:

Two assays for microscopy-based high-throughput screening of host factors involved in *Brucella* infection are described. The entry assay detects host factors required for *Brucella* entry and the endpoint assay those required for intracellular replication. While applicable for alternative approaches, siRNA screening in HeLa cells is used to illustrate the protocols.

LONG ABSTRACT:

Brucella species are facultative intracellular pathogens that infect animals as their natural hosts. Transmission to humans is most commonly caused by direct contact with infected animals or by ingestion of contaminated food and can lead to severe chronic infections.

Brucella can invade professional and non-professional phagocytic cells and replicates within endoplasmic reticulum (ER)-derived vacuoles. The host factors required for *Brucella* entry into host cells, avoidance of lysosomal degradation, and replication in the ER-like compartment remain largely unknown. Here we describe two assays to identify host factors involved in *Brucella* entry and replication in HeLa cells. The protocols describe the use of RNA interference, while alternative screening methods could be applied. The assays are based on the detection of fluorescently labeled bacteria in fluorescently labeled host cells using automated wide-field microscopy. The fluorescent images are analyzed using a standardized image analysis pipeline in CellProfiler which allows single cell-based infection scoring.

In the endpoint assay, intracellular replication is measured two days after infection. This allows bacteria to traffic to their replicative niche where proliferation is initiated around 12 h after bacterial entry. *Brucella* which have successfully established an intracellular niche will thus have strongly proliferated inside host cells. Since intracellular bacteria will greatly outnumber individual extracellular or intracellular non-replicative bacteria, a strain constitutively expressing GFP can be used. The strong GFP signal is then used to identify infected cells.

In contrast, for the entry assay it is essential to differentiate between intracellular and extracellular bacteria. Here, a strain encoding for a tetracycline-inducible GFP is used. Induction of GFP with simultaneous inactivation of extracellular bacteria by gentamicin enables the differentiation between intracellular and extracellular bacteria based on the GFP signal, with only intracellular bacteria being able to express GFP. This allows the robust detection of single intracellular bacteria before intracellular proliferation is initiated.

INTRODUCTION:

Brucella species are gram-negative, facultative intracellular pathogens belonging to the class of α -Proteobacteria. They cause abortions and infertility in their natural hosts such as cattle, goats, or sheep resulting in severe economic losses in endemic areas. Brucellosis is one of the most important zoonotic diseases worldwide causing over half a million new human infections annually¹. Transmission to human is most commonly caused by direct contact with infected animals or by ingestion of contaminated food such as unpasteurized milk. Symptoms of the febrile disease are unspecific, which causes difficulties in the diagnosis of brucellosis. If untreated, patients can develop a chronic infection with more severe symptoms such as arthritis, endocarditis, and neuropathy².

On the cellular level *Brucella* is able to invade phagocytic and non-phagocytic cells and replicates within an intracellular compartment known as the *Brucella*-containing vacuole (BCV). Internalization of bacteria requires actin cytoskeleton rearrangements by Rac, Rho, and direct activation of Cdc42³. Inside the eukaryotic host cell, the BCV traffics along the endocytic pathway and despite the interaction with lysosomes, bacteria manage to avoid degradation⁴. Acidification of the BCV by the vesicular ATPase is required to induce the expression of the bacterial type IV secretion system (T4SS)⁵. It is believed that bacterial effectors secreted by the T4SS are essential for *Brucella* to establish its replicative niche, since deletion of the T4SS⁶ or inhibition of the vesicular ATPase lead to defects in the establishment of the intracellular niche⁷. Bacteria do not replicate during the phase of trafficking until they reach an ER-derived vacuolar compartment⁸. Once intracellular proliferation occurs, the BCV is found in close association with ER markers such as calnexin and glucose-6-phosphatase⁶.

The molecular mechanisms by which *Brucella* enters cells, avoids lysosomal degradation, and finally replicates in an ER-like compartment remain largely unknown. Host factors involved in different steps of infection have mainly been identified by targeted approaches or a small scale small interfering RNA (siRNA) screen performed in *Drosophila* cells⁹. These have shed light on the contribution of individual host factors during *Brucella* infection but we are still far from a comprehensive understanding of the entire process.

Here, protocols that allow the identification of human host factors using large-scale RNA interference (RNAi) screening in combination with automated wide-field fluorescence microscopy and automated image analysis are presented. Reverse siRNA transfection of HeLa cells is performed as described earlier^{10,11} with minor modifications. The endpoint assay covers a large part of the *Brucella* intracellular lifecycle except the egress and the infection of neighboring cells. To further characterize the hits identified in the endpoint assay, a modified protocol to identify factors involved in early steps of the infection is used.

A *Brucella abortus* strain that constitutively expresses GFP is used for the endpoint assay where bacteria are allowed to infect cells for two days. During this time, bacteria enter cells, traffic to the ER-derived replicative niche, and replicate in the peri-nuclear space. The high levels of GFP signal can then be used to reliably detect individual cells which contain replicating bacteria.

To study *Brucella* entry in a high-throughput assay, it is important to be able to distinguish between intracellular and extracellular bacteria. The method presented here circumvents differential antibody staining of intracellular and extracellular bacteria. It is based on a *Brucella* strain expressing a tetracycline-inducible GFP in combination with constitutive expression of dsRed. The presence of a constitutive dsRed marker allows identification of all bacteria that are present in the sample. GFP expression is induced by the addition of the non-toxic tetracycline analog anhydrotetracycline (aTc) simultaneously with inactivation of extracellular bacteria by gentamicin (Gm). While the cell-impermeable antibiotic Gm kills extracellular bacteria, aTc can enter the host cell and induce GFP expression selectively in intracellular bacteria. This dual reporter allows the robust separation of single intracellular bacteria (GFP and dsRed signal) from extracellular bacteria (only dsRed signal) using wide-field microscopy. In order to reach detectable GFP expression by intracellular *Brucella* we have found that 4 h of induction by aTc results in a reliable signal. Similar induction schemes to selectively express GFP in intracellular bacteria has been used previously to study intracellular *Shigella*¹².

PROTOCOL:

Note: All work with live *Brucella abortus* strains must be performed inside a biosafety level 3 (BSL3) laboratory considering all required regulations and safety precautions.

1. Preparation of screening plates and culturing of bacteria and cells

1.1) Preparation of *Brucella abortus* starter cultures

1.1.1) Streak *Brucella abortus* 2308 (*B. abortus*) from -80°C milk stock on a Tryptic Soy Agar (TSA) plate containing 50 µg/ml kanamycin (TSA/Km). Incubate plate for 3-4 days at 37°C. Use strain *Brucella abortus* 2308 pJC43 (*apHT::GFP*)¹³ for the endpoint assay and strain *Brucella abortus* pAC042.08 (*apht::dsRed*, *tetO::tetR-GFP*) for the entry assay.

Note: The smooth lipopolysaccharide (LPS) of *Brucella abortus* is an important virulence determinant but rough mutants can occur at relatively high frequencies¹⁴. We thus recommend testing the status of the culture strain before starting with this experiment.

1.1.2) Restreak bacteria on four TSA/Km plates covering the full plate and incubate for 2-3 days at 37°C.

1.1.3) Using a disposable plastic loop, transfer and resuspend bacteria from the TSA/Km plates in 10 ml 10% autoclaved skim milk. Make sure that the bacteria are well resuspended to ensure consistent bacterial concentrations in all starter cultures.

1.1.4) Aliquot 250 µl of the bacterial suspension into 2 ml screw cap tubes and freeze at -80°C.

1.1.5) Thaw an aliquot of the starter culture and transfer 25 µl, 50 µl, and 100 µl of the bacterial starter culture to separate 250 ml screw cap bottles with 50 ml Tryptic Soy Broth (TSB) containing 50 µg/ml kanamycin (TSB/Km). Seal the bottles with parafilm.

1.1.6) Incubate the bottles overnight on an orbital shaker at 100 rpm and 37°C.

1.1.7) Measure the optical density at 600 nm (OD₆₀₀) to determine the volume of starter culture required to reach an OD₆₀₀ = 0.8-1.1 after overnight culture.

1.2) Preparation of HeLa cells

1.2.1) Grow HeLa cells in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (DMEM/10% FCS) in a 37°C humid incubator with 5% CO₂. Grow cells to 80% confluency.

1.3) Prepare the screening plates with siRNAs.

1.3.1) Dilute siRNAs in RNase-free water to a final concentration of 0.32 µM. Transfer 5 µl/well to black clear-well flat-bottom 384-well plates.

1.3.1.1) Use columns 1, 2, 23, and 24 for standard controls. For negative controls, use non-targeting siRNA (scrambled) and mock wells without siRNAs (transfection reagent only). For transfection controls, use an siRNA that induces cell death (KIF11), as well as positive controls of known host factors involved in *Brucella* infection (e.g. ARPC3, a component of the Arp2/3 complex involved in actin polymerization). Transfer commercially available siRNA libraries or custom siRNAs to the remaining wells.

1.3.2) Seal plates with peelable aluminum foils. Store plates at -20°C.

Note: Plates can be stored at -20°C for at least 6 months and up to years depending on the manufacturer's recommendations.

2. Reverse siRNA transfection

2.1) Thaw a black 384-well plate by centrifugation at 300 x g for 20 min at room temperature.

Note: This will bring down all the liquid which might adhere to the lid or the side of the wells.

2.2) Prepare the transfection medium by diluting the transfection reagent 1:200 in DMEM without FCS at room temperature. Prepare the transfection medium no longer than 20 min prior to use. Carefully mix the solution before use.

2.3) Add 25 µl transfection medium to each well using a reagent dispenser and mix the solutions by moving the plate back and forth.

2.4) Incubate the plate for 1 h at room temperature to allow siRNA/transfection reagent complex formation.

2.5) In the meantime, prepare HeLa cells by washing the sub-confluent cells of a 75 cm² flask once with 2.5 ml 0.05% trypsin-EDTA in PBS (trypsin).

2.6) Add 1.5 ml fresh trypsin and transfer the flask to 37°C for 2-3 min until the cells round up.

2.7) Resuspend the cells in 10 ml pre-warmed DMEM/16% FCS.

2.8) Count cells using an automated cell counter and prepare a cell suspension of 10,000 cells/ml in DMEM/16% FCS.

2.9) Add 50 µl cell suspension to each well using a reagent dispenser resulting in 500 cells/well.

2.10) Move the plate back and forth to achieve even distribution of the cells. Leave the plate at room temperature for 5-10 min to allow for the cells to settle down.

2.11) Seal the plate with parafilm and incubate it for 72 h on a pre-warmed aluminum plate in a 37°C humid incubator with 5% CO₂. Note: The pre-warmed aluminum plate allows equal temperature distribution throughout the whole 384-well plate.

3. Infection and fixation

3.1) One day prior to infection, inoculate the amount determined in 1.1.7 of *B. abortus* starter culture in 50 ml TSB/Km medium in a 250 ml screw cap bottle. Seal the bottle with parafilm and grow bacteria overnight at 37°C and 100 rpm on an orbital shaker to OD₆₀₀ = 0.8-1.1.

3.2) Measure OD₆₀₀ of the bacterial culture and prepare the infection medium by diluting the bacterial culture in DMEM/10% FCS to achieve the desired multiplicity of infection (MOI).

Note: To obtain an infection rate of 5-10% in HeLa cells, an MOI of 10,000 is used. A MOI titration curve should be performed for each cell type to obtain optimal infection rates.

3.3) Exchange the transfection medium of the 384-well plate with 50 µl of the infection medium using an automated plate washer.

3.4) Centrifuge the 384-well plate at 400 x *g* and 4°C for 20 min.

Note: This helps to synchronize the infection process.

3.5) Seal the plate with parafilm and incubate it on a pre-warmed aluminum plate in a 37°C humid incubator with 5% CO₂ for 4 h.

3.6) Wash the cells with DMEM/10% FCS containing 100 µg/ml Gm to inactivate extracellular bacteria using the automated plate washer (use 200 µl/well overflow wash). Note: During an overflow wash, the automated plate washer simultaneously dispenses and aspirates medium.

3.7) For the entry assay, wash cells a second time with DMEM/10% FCS containing 100 µg/ml Gm and 100 ng/ml aTc using the automated plate washer (use 200 µl/well overflow wash).

3.8) Seal the plate with parafilm and return it to a pre-warmed aluminum plate in the 37°C humid incubator with 5% CO₂ for another 40 h or 4 h, for the endpoint assay and the entry assay, respectively.

3.9) For fixation, wash the wells with PBS using the automated plate washer (use 200 µl/well overflow wash).

3.10) Exchange PBS with 50 µl 3.7% paraformaldehyde in 0.2 M HEPES at pH 7.4 (PFA) using the automated plate washer. Note: Caution: PFA is toxic by inhalation, in contact with skin and if swallowed.

3.11) Incubate for 20 min at room temperature.

3.12) Exchange the fixation medium with 50 µl PBS using the automated plate washer. Note: The samples are now ready to be taken out from the BSL3 if needed. Caution: Removal of any sample from a BSL3 facility is subject to risk assessment and validation of the procedure and depends on the applicable regulations for biosafety.

4. Staining

4.1) Wash cells twice with 50 µl PBS.

4.2) Permeabilize cells in 50 µl 0.1% Triton-X-100 in PBS for 10 min.

4.3) Wash cells three times with PBS. Add 20 µl of PBS containing 1 µg/ml DAPI (4',6-diamidino-2-phenylindole) and incubate for 30 min at room temperature.

4.4) Wash cells three times in PBS and protect the staining from light with an aluminum foil.

5. Imaging

5.1) Set up the automated wide-field microscope for image acquisition. Note: The settings for a Molecular Device ImageXpress microscope using MetaXpress software are described below. Alternative 2D wide-field microscopy devices with appropriate hardware specifications and imaging software can be used (see Table of Material/Equipment for details).

5.1.1) Select 10 X objective, camera binning = 1, gain = 1.

5.1.2) Select the plate format corresponding to the 384-well plate.

5.1.3) Acquire 9 sites per well.

5.1.4) Use “Enable laser based focus” and “Focus on plate and well bottom”.

5.1.5) Set “First well” as the initial well for finding the sample and “All sites” for site autofocus.

5.1.6) Use the DAPI channel to manually set Z-Offset for focus.

5.1.7) Manually select the Z-Offset from DAPI for all other channels.

5.1.8) Manually correct the exposure time for all channels to ensure a wide dynamic range with low overexposure.

5.1.8.1) Acquire an image of a representative site using the auto-exposure function. Use this exposure time to image 3-5 sites throughout the plate and adjust the exposure time manually until the brightest pixels reach ~ 80% of the maximal brightness over all sites.

5.1.9) Image the full plate using the DAPI and GFP channels for the endpoint assay. For the entry assay select the RFP channel in addition.

6. Automated image analysis

Note: CellProfiler 2¹⁵ is employed as image analysis software to segment cellular and bacterial objects and perform automated measurements within the identified objects. The software provides image analysis algorithms in individual modules, which can be combined into a pipeline that will execute the modules consecutively on all images, to automatically perform a specific image analysis task. To follow the protocol, install CellProfiler 2.1.1 or a newer version. Then, load the provided pipeline and follow the instructions below to adjust the required parameters within the modules. A description of the individual modules of all pipelines can be found in the Supplemental Files.

Note: Two separate pipelines are used for each assay. The first pipeline calculates a shading model, which is used by the second pipeline to correct images prior to analysis. Shading correction is applied to the images to reduce the effects of an inhomogeneous light path from the microscope. Computing the shading model in the first pipeline is a lengthy process, but the results will be of higher accuracy.

6.1) Endpoint assay

6.1.1) Calculate the shading model

6.1.1.1) Go to the Menu “File”, select “Import” → “Pipeline from File...”, and select the provided pipeline “BrucellaShadingCorrectionPart1-Ver007”. When asked whether to convert the legacy pipeline, answer “Don’t convert”.

6.1.1.2) Go to “Output” → “View output settings” and select an input folder with the images and an output folder for the resulting shading models.

6.1.1.3) In Module (1) “LoadImages”, adjust the name of the “Text that these images have in common” to match the image names.

6.1.1.4) Make sure that none of the modules show their display prior to running the full analysis by unchecking all eye symbols next to the modules. Note: This significantly decreases required computational resources while processing the pipeline.

6.1.1.5) Press “Analyze Images” to start the calculation of the shading model.

6.1.2) Run image analysis

6.1.2.1) Go to the Menu “File”, select “Import” → “Pipeline from File...”, and select the provided pipeline “BrucellaEndpointWithShadingCorrectionPart2-Ver007”. When asked whether to convert the legacy pipeline, answer “Don’t convert”.

6.1.2.2) Go to “Output” → “View output settings” and select an input folder with the images and an output folder for the resulting spreadsheet.

6.1.2.3) In module (1) “LoadImages”, adjust the name of the “Text that these images have in common” to match the image names.

6.1.2.4) In module (2) “LoadSingleImage”, load the shading models calculated under 6.1.1 by selecting the location and filenames of the two shading models.

6.1.2.5) In modules (4) – (5) “ImageMath”, adjust the parameter “Multiply the first image by” matching the bit depth of the microscope camera. For 8 and 16bit images set the parameter to “1.0”, for 12bit images set the parameter to “16.0”.

6.1.2.6) In module (9) “ImageMath” adjust the parameter “Multiply the second image by” to a value (start with 0.25) that will suppress the *Brucella* signal in the DAPI staining without suppressing the Nuclei.

6.1.2.6.1) Click “Start Test Mode”, then activate the pause symbol and the display function for module (9) by clicking on the corresponding icons of the module. Note: The pause symbol will appear in yellow, while the eye symbol will show an open eye if activated.

6.1.2.6.2) Click “Run” to run the analysis up to module (9) with the active pause symbol. To test the values for the module, press “Step”.

6.1.2.6.3) Right-click the newly opened image and set “Image contrast” to “Log normalized”. Repeatedly step back to module (9), adjust the parameter “Multiply the second image by”, and step over the module again, until the *Brucella* signal is fully suppressed while at the same time black areas that indicate over-subtraction are minimized in the result image.

6.1.2.7) In module (10) “IdentifyPrimaryObjects”, set the parameter “Lower bound on threshold” high enough (start with zero) so that nuclei are segmented and background is ignored.

6.1.2.7.1) To identify a good value for module (10), step over the module and display the input image as explained in steps 6.1.2.6.1 and 6.1.2.6.2.

6.1.2.7.2) Right-click the input image and display the histogram. Note: The peak of the histogram typically indicates background.

6.1.2.7.3) Starting from background intensity increase the parameter until a good segmentation of nuclei is achieved as displayed in the output image.

Note: Setting the threshold higher than background intensity is especially important for empty sites.

6.1.2.8) In module (15) “FilterObjects”, adjust the parameter “Minimum value” so that cells with clearly visible *Brucella* in the nucleus are kept, and all others are filtered away. In this step, ignore *Brucella* outside the nucleus.

6.1.2.8.1) To identify a good value, step over the module and display the output image as explained in steps 6.1.2.6.1 and 6.1.2.6.2. Start from zero, which identifies all cells as infected, and increase the value by small steps until only cells with clearly visible *Brucella* at the nucleus are kept.

6.1.2.9) In module (16) “FilterObjects”, adjust the parameter “Minimum value” so that cells with clearly visible *Brucella* at the perinucleus are kept, and all other cells are filtered away. Use a similar approach as in the previous module.

6.1.2.10) In module (17) “FilterObjects”, adjust the parameter “Minimum value” so that cells with clearly visible *Brucella* at the Voronoi cell body are kept, and all other cells are filtered away. Use a similar approach as in the previous module. Note: The Voronoi cell body is a radial extension of the nucleus by 25 pixels with no overlap with neighboring Voronoi cell bodies.

6.1.2.11) Exit the test mode and make sure that none of the modules show their display prior to running the full analysis by unchecking all eye symbols next to the modules. Note: This significantly decreases required computational resources while processing the pipeline.

6.1.2.12) Press “Analyze Images” to start the analysis.

6.1.2.13) When the analysis has finished, inspect the resulting PNG images as well as the CSV sheet to ensure that the analysis worked reliably throughout the plate.

6.2) Entry assay

6.2.1) Calculate the shading model

6.2.1.1) Go to the Menu “File”, select “Import” → “Pipeline from File...”, and select the provided pipeline “BrucellaShadingCorrectionPart1-Ver007”. When asked whether to convert the legacy pipeline, answer “Don’t convert”.

6.2.1.2) Go to “Output” → “View output settings” and select an input folder with the images and an output folder for the resulting shading models.

6.2.1.3) In module (1) “LoadImages”, adjust the name of the “Text that these images have in common” to match the image names.

6.2.1.4) Make sure that none of the modules show their display prior to running the full analysis by unchecking all eye symbols next to the modules. Note: This significantly decreases required computational resources while processing the pipeline.

6.2.1.5) Press “Analyze Images” to start the calculation of the shading model.

6.2.2) Run image analysis

6.2.2.1) Go to the Menu “File”, select “Import” → “Pipeline from File...”, and select the provided pipeline “BrucellaEntryWithShadingCorrectionPart2-Ver007”. When asked whether to convert the legacy pipeline, answer “Don’t convert”.

6.2.2.2) Go to “Output” → “View output settings” and select an input folder with the images and an output folder for the resulting spreadsheet.

6.2.2.3) In module (1) “LoadImages”, adjust the name of the “Text that these images have in common” to match the image names.

6.2.2.4) In module (2) “LoadSingleImage”, load the shading models calculated under 6.2.1 by selecting the location and filenames of the two shading models.

6.2.2.5) In modules (4) – (5) “ImageMath”, adjust the parameter “Multiply the first image by” matching the bit depth of the microscope camera. For 8 and 16bit images set the parameter to “1.0”, for 12bit images set the parameter to “16.0”.

6.2.2.6) In module (6) “IdentifyPrimaryObjects”, set the parameter “Lower bound on threshold” high enough that only nuclei are segmented and background is ignored.

6.2.2.6.1) To identify a good value for module (6), step over the module and display the input image as explained in steps 6.1.2.6.1 and 6.1.2.6.2.

6.2.2.6.2) Right-click the input image and display the histogram. Note: The peak of the histogram typically indicates background.

6.2.2.6.3) Starting from background intensity increase the parameter until a good segmentation of nuclei is achieved as displayed in the output image. Note: Setting the threshold above background is important for empty sites.

6.2.2.7) In module (8) “IdentifyPrimaryObjects”, set the parameter “Lower bound on threshold” high enough that only *Brucella* are segmented and background is ignored.

6.2.2.7.1) To identify a good value for module (8), step over the module and display the input image as explained in steps 6.1.2.6.1 and 6.1.2.6.2.

6.2.2.7.2) Right-click the input image and display the histogram. Note: The peak of the histogram typically indicates background.

6.2.2.7.3) Starting from background intensity increase the parameter until good segmentation of *Brucella* as displayed in the output image is achieved. Note: Setting the threshold above background is important for empty sites.

6.2.2.8) In module (11) “FilterObjects”, adjust the parameter “Minimum value” so that background and artefacts are filtered away, and only *Brucella* colonies are kept.

6.2.2.8.1) To identify a good value, step over the module and display the output image as explained in steps 6.1.2.6.1 and 6.1.2.6.2. Start with the value selected in 6.2.2.7 and stepwise increase it until only *Brucella* objects are kept.

6.2.2.9) Exit the test mode and make sure that none of the modules show their display prior to running the full analysis by unchecking all eye symbols next to the modules. Note: This significantly decreases required computational resources while processing the pipeline.

6.2.2.10) Press “Analyze Images” to start the analysis.

6.2.2.11) When the analysis has finished, inspect the resulting PNG images as well as the CSV sheet to ensure that the analysis worked well throughout the plate.

7. Infection scoring

Note: siRNAs which have a significant impact on cell viability have to be considered with caution, since this can promote false positive discoveries. An altered cell number affects the actual MOI and targeting of essential genes can have pleiotropic effects on pathogen infection. While the incomplete depletion by siRNAs allows for the study of essential genes, such targets have to be validated by alternative methods (e.g. pharmaceutical interference) to corroborate their role as host factors during infection.

7.1) Endpoint assay

7.1.1) Open the CSV sheet generated in step 6.1.2.12 and calculate a per well infection rate by dividing the number of infected cells (CellProfiler readout: “Count_InfectedCells”) by the total number of cells (CellProfiler readout: “CountNuclei”). If multiple sites per well have been imaged, first summarize all sites for each well, to build a per-well number of infected cells and a per-well total number of cells.

7.1.2) Perform Z scoring normalization to account for plate-to-plate variations if the plates contain sufficient non-hit siRNAs. Assume a sufficient number of non-hit siRNAs per plate if full libraries are screened.

$$Zscore = \frac{\text{Infection rate (well)} - \text{mean infection rate (plate)}}{\text{standard deviation (plate)}}$$

7.2) Entry assay

7.2.1) Open the CSV sheet generated in step 6.2.2.10 and calculate a per well infection rate by dividing the number of infected cells (CellProfiler readout: “Count_InfectedCells”) by the total number of cells (CellProfiler readout: “CountNuclei”). If multiple sites per well have been imaged, first summarize all sites for each well, to build a per-well number of infected cells and a per-well total number of cells.

7.2.2) To quantify the bacterial load of infected cells, estimate the average area occupied by intracellular bacteria per infected cell. To this end, divide the integrated area of all intracellular bacteria overlapping with cells (CellProfiler readout: “AreaOccupied_AreaOccupied_TrueIntPathogenInCells”) by the number of infected cells in this site. To account for artefacts, use the median of all sites of this readout as the well readout.

Note: If this assay is used as a follow up assay containing a large number of genes involved in *Brucella* infection, do not apply Z score normalization. Instead, perform normalization using mock wells as a reference to account for plate-to-plate variation.

REPRESENTATIVE RESULTS:

Figure 1A shows an example of image analysis used to automatically identify infected cells in the endpoint assay. Nuclei of HeLa cells stained with DAPI were identified, a peri-nucleus of 8 pixels width surrounding the nucleus, and a Voronoi cell body by extension of the nucleus by 25 pixels were calculated. Since bacteria mainly proliferate in the peri-nuclear space, the GFP intensity in this area of the cell is the most robust measurement to discriminate between infected and non-infected cells. In some cases, bacteria are found to proliferate outside of the peri-nucleus or overlay to a large extent with the nucleus. Therefore, these two additional objects were also considered for the identification of infected cells. Segmentation as well as GFP intensity measurements were performed with the image analysis software CellProfiler 2.

Brucella requires actin rearrangements for successful invasion of host cells³. Thus, depletion of actin remodeling components is a suitable positive control for siRNA screening. We have found that siRNA mediated depletion of Arp2/3 complex components, which are involved in actin polymerization, strongly inhibits *Brucella* infection of HeLa cells (unpublished data). Thus, knockdown of ARPC3 was used as a positive control. As seen in Figure 1B, depletion of ARPC3 reduced the number of cells that show proliferating bacteria two days after infection. Applying the automated image analysis pipeline to these images allowed the quantification of the observed effect (Figure 1C). The data which are shown here originate from the control wells of a genome-wide siRNA screen. Z scoring was applied to account for plate-to-plate variations.

Figure 2A illustrates the image analysis used to identify infected cells and measure the intracellular bacterial load in the entry assay. In contrast to the endpoint assay, the entry assay uses bacterial segmentation and a Voronoi cell body as cellular object. Only the GFP signal of intracellular *Brucella* was used to segment the pathogen in this image analysis pipeline. Intracellular bacteria were defined by a minimal size of 2 pixels and a GFP intensity which exceeds the dim GFP background intensity of extracellular bacteria. A cell was considered infected if at least one intracellular bacterial object overlapped with its Voronoi cell body. Furthermore, the bacterial load was estimated by calculating the average area of infected cells that is covered by intracellular bacteria.

As for the endpoint assay, depletion of ARPC3 showed a reduction in *Brucella* infection in the entry assay compared to cells treated with a control siRNA (Figure 2B). Quantification of the infection rate confirmed that the number of infected cells (Figure 2C) as well as the number of intracellular bacteria in infected cells (Figure 2D) was reduced by depletion of ARPC3.

Figure Legends:

Figure 1: Analysis of HeLa cells infected by *B. abortus* expressing GFP in the endpoint assay. A) Endpoint assay: Fluorescence image showing an example of the endpoint assay (green = *B. abortus* expressing GFP, blue = nuclei stained with DAPI, scale bar = 50 μ m). GFP expressing *B. abortus* were allowed to enter HeLa cells for 4 h followed by killing of extracellular bacteria by Gm. Further incubation for 40 h allowed bacteria to replicate inside HeLa cells. Automated image analysis: A CellProfiler pipeline was used to segment DAPI stained nuclei followed by

calculation of the peri-nucleus (non-overlapping ring of 8 pixels surrounding the nucleus) and Voronoi cell body (non-overlapping radial extension of the nucleus by 25 pixels), both shown in red. A cell was considered infected if the integrated GFP intensity in at least one cellular compartment (nucleus, peri-nucleus, Voronoi cell body) exceeded the corresponding threshold. **B)** Representative images of HeLa cells infected with *B. abortus* expressing GFP 44 h after infection. Cells were either transfected with a scrambled (non-targeting) siRNA control or a siRNA targeting ARPC3. Scale bar = 50 μ m. **C)** Quantification of infection of HeLa cells depleted for ARPC3. The data are represented as bar graphs (bar = mean; Z score normalization; error bars = standard error of the mean; *** $p < 0.001$; Mann-Whitney test; $n = 7$).

Figure 2: Quantification of infection of HeLa cells by *B. abortus* expressing TetR-GFP in the entry assay. **A)** Entry assay: Fluorescence image showing an example of entry assay (yellow = intracellular *B. abortus* showing dsRed and GFP signal, red = extracellular *B. abortus* showing dsRed signal, blue = nuclei stained with DAPI, scale bar = 50 μ m). *B. abortus* expressing dsRed and TetR-GFP were allowed to enter HeLa cells for 4 h followed by killing of extracellular bacteria and simultaneous induction of GFP in intracellular bacteria with ATc for 4 h. Automated image analysis: A CellProfiler pipeline was used to detect DAPI stained nuclei followed by calculation of a Voronoi cell body by radial extension of the nucleus by 25 pixels (shown in white). Bacteria were segmented based on the GFP signal. A cell was considered infected if its Voronoi cell body overlapped with at least one segmented bacterial object of sufficient size and GFP intensity. The bacterial load in infected cells is illustrated by the integration of the area of all segmented bacterial objects with its Voronoi cell body (Unit = pixels; NaN = no number is calculated in non-infected cells). **B)** Example images illustrating a decrease of intracellular *B. abortus* (shown by the number of yellow bacteria) in cells transfected with a siRNA targeting ARPC3 compared to control cells treated with scrambled siRNA. The number of infected cells as well as the average number of intracellular bacteria in infected cells is reduced upon ARPC3 knock down. **C)** Quantification of infection rate in HeLa cells depleted for ARPC3. The data are represented as bar graphs (bar = mean; normalization to mock; error bars = standard error of the mean; * $p < 0.05$; Mann-Whitney test; $n = 4$). **D)** Quantification of bacterial load of infected HeLa cells depleted for ARPC3. The data are represented as bar graphs (bar = mean; normalization to mock; error bars = standard error of the mean; * $p < 0.05$; Mann-Whitney test; $n = 4$).

Supplemental File: CP2 pipeline - shading correction.

Supplemental File: CP2 pipeline - Endpoint assay

Supplemental File: CP2 pipeline - Entry assay

Supplemental File: Description of Modules

DISCUSSION:

Bacterial pathogens have evolved numerous strategies to manipulate eukaryotic host cells to

their benefit. Pathogens causing acute infections often show rapid proliferation which is accompanied by significant alarming of the immune system and loss of viability of infected cells. In contrast, *Brucella* and other pathogens that cause chronic infections manage to establish long-lasting interactions within host cells. Therefore, bacteria need to fine tune host cell functions to their benefit without disrupting cellular homeostasis. A common strategy to identify the host cell factors involved in an infection process on a genome scale is the use of systematic gene depletion for example by siRNAs coupled to a functional readout of infection. Here we present protocols suitable for high-content, high-throughput screening based on detection of fluorescent bacteria.

An important aspect of successful large-scale screening involves a robust automated image analysis and infection scoring. The methods described here rely on the detection of a cell nucleus by DAPI staining which is generally very robust, and the definition of an area of interest where the pathogen signal is measured. The endpoint assay makes use of the detection of proliferating bacteria in the peri-nucleus, nucleus, and Voronoi cell body. This allows reliable identification of the infected cells. Furthermore, it allows defining different thresholds for the individual compartments. While the thresholds can be set relatively low in the nucleus and peri-nucleus, higher thresholds should be employed for the Voronoi cell body. This helps to avoid the detection of GFP signal from neighboring cells which is important since the endpoint assay lasts for five days during which HeLa cells have proliferated and grown to confluency.

The entry assay in contrast only lasts for three days and HeLa cells have thus not grown to confluency. Here, only a Voronoi cell body is chosen as cellular object for three main reasons. First, early during infection some bacteria might not yet have reached the nuclear or peri-nuclear area and may not be detected by measurements within these objects. Second, in contrast to the endpoint assay, bacteria have not started intracellular replication. Thus, using the Voronoi cell body object avoids missing individual bacteria which are located further away from the nucleus. Third, the entry assay makes use of quantification of the bacterial load which makes it desirable to cover as many bacteria as possible, which is more reliably achieved by using a Voronoi cell body compared to a peri-nucleus.

In the entry assay, the dsRed fluorescent marker which is constitutively expressed in all bacteria is not directly used for infection scoring. However, it serves as important quality control. Visual inspection of images gives a good indication whether bacteria that are distant from any nucleus and thus outside cells show no or only very weak GFP signal compared to bacteria in close proximity of a nucleus, indicative of an intracellular location. This is an important control to ensure that induction of GFP by aTc as well as inactivation of extracellular bacteria by Gm was efficient. Here, it has to be considered that in the absence of aTc bacteria express very low levels of GFP. In addition, extracellular bacteria (red signal and no/weak GFP signal) that overlap with a HeLa cell body can provide further information. In this case, bacteria could either be bound to the cell surface or they were internalized but failed to induce the GFP signal, indicative of very rapid killing of bacteria inside a host cell. These hypotheses can then be further tested with alternative assays such as counting of colony forming units (CFU) on TSA plates.

A further advantage of using a tetracycline inducible system over differential antibody staining to visualize intracellular bacteria is its applicability for live cell imaging. Depletion of host factors potentially affects various stages of the infection process which can be resolved by quantifying the dynamics of intracellular stages. To this end, the tetracycline inducible system can be used with live cell imaging to measure the onset of replication or to provide the information of the percentage of intracellular bacteria that initiate replication.

While the protocol described here is tailored for the use of siRNAs it can be easily adapted to other screening strategies, such as CRISPR-Cas9, small hairpin RNA (shRNAs), and chemical library screening. This would only require basic experimental modifications whereas the image analysis pipeline can be used directly regardless of the screening strategy. Moreover, the assay design is not restricted to *Brucella* and is readily adapted to other intracellular pathogens¹⁰. As such, this protocol is widely applicable for screening host factors involved in pathogen mediated-infection of host cells.

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DISCLOSURES:

The authors declare no conflicts of interest.

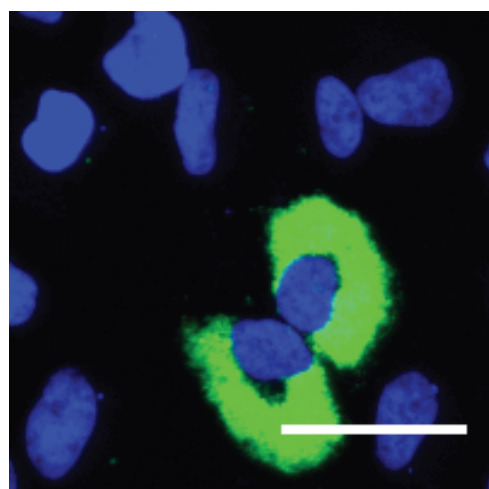
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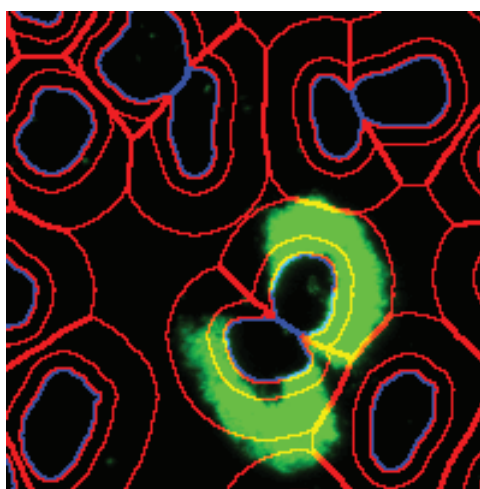
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A

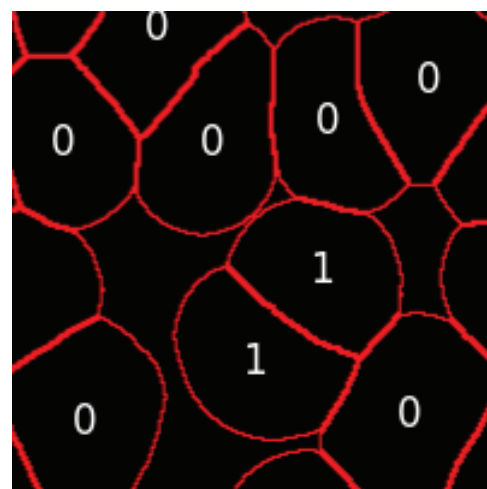
Original image



Object segmentation



Detection of infection



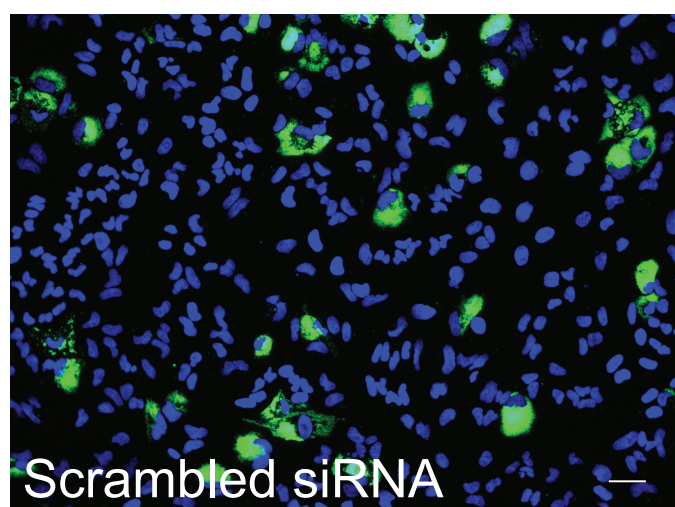
Nuclei (DAPI)

Brucella (GFP)

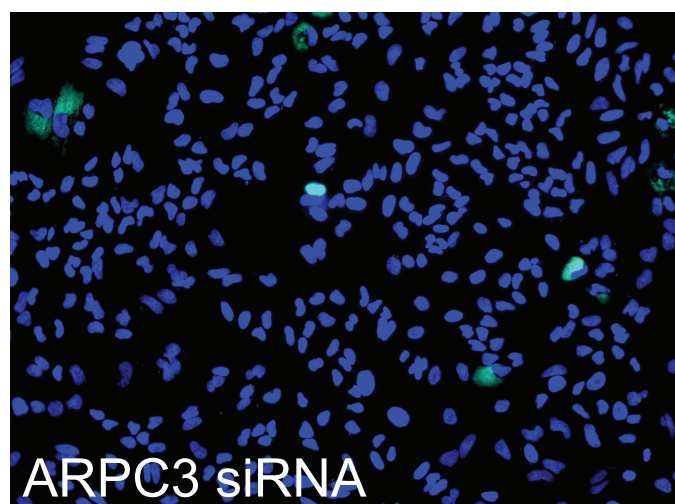
Peri-nucleus and Voronoi cell body

1: infected cell

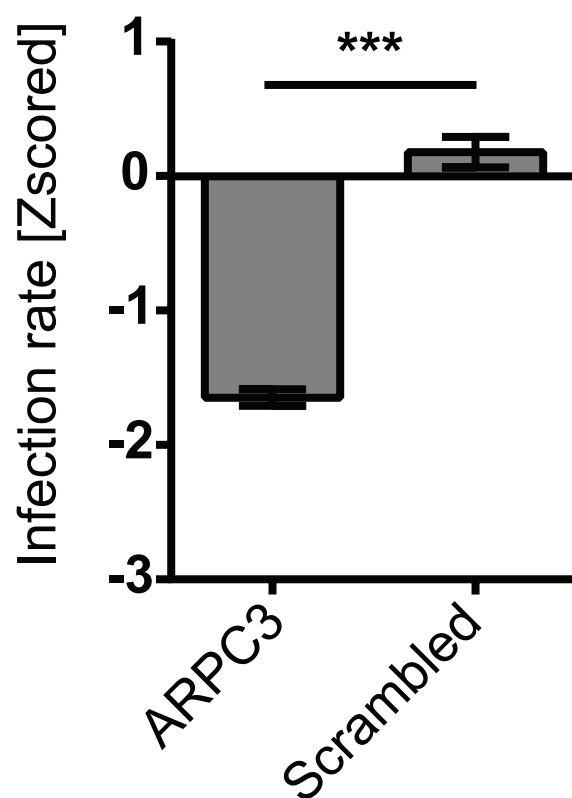
0: non-infected cell

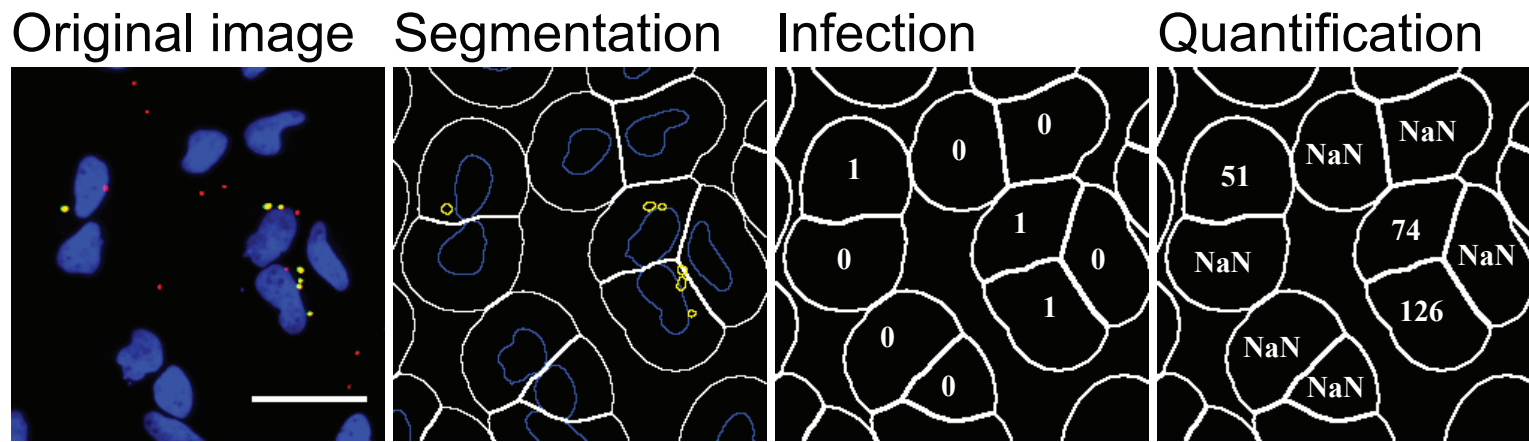
B

Scrambled siRNA

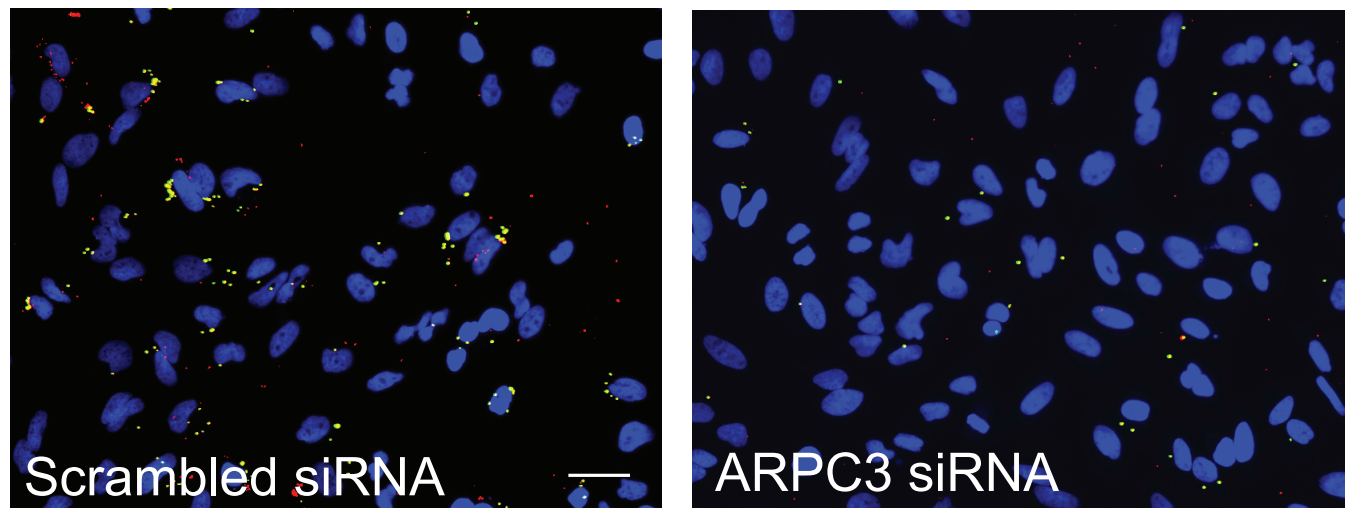
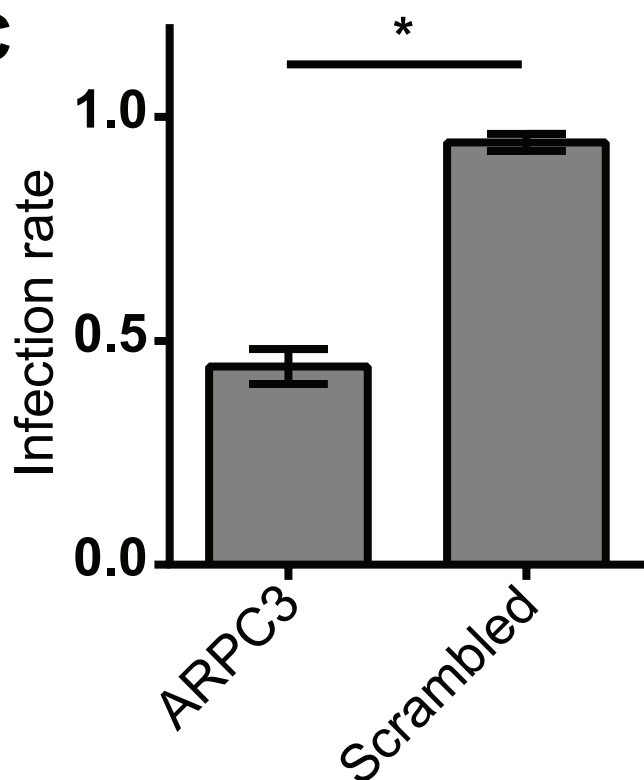
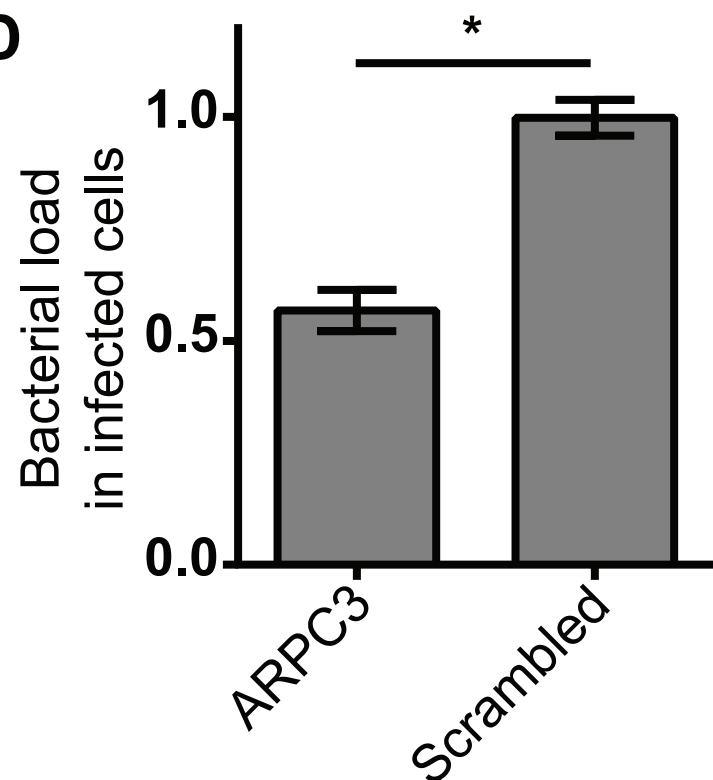


ARPC3 siRNA

C

A

Nuclei (DAPI)

All *Brucella* (dsRed)Intracellular *Brucella* (GFP + dsRed)**B****C****D**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Tryptic Soy Agar (TSA)	BD	236950	
Tryptic Soy Broth (TSB)	Fluka	22092	
Kanamycin sulfate	Sigma-Aldrich	60615	
Skim milk			
250 ml screw cap bottle	Corning	8396	
DMEM	Sigma-Aldrich	D5796	
Fetal Calf Serum (FCS)	Gibco	10270	Heat-inactivated
Trypsin-EDTA (0.5%)	Gibco	15400-054	10x stock solution, dilute 1:10 in PBS
Scepte 2.0 Cell Counter	Merck Milipore	PHCC20060	Alternative cell counting devices can be used.
Greiner CELLSTAR 384-well plate	Sigma-Aldrich	M2062	
Peelable aluminum foil	Costar	6570	
Reagent dispenser: "Multidrop 384 Reagent Dispenser"	Thermo Scientific	5840150	Alternative reagent dispenser can be used.
Transfection reagent: "Lipofectamine RNAiMAX	Invitrogen	13778-150	

Automated plate washer: "Plate washer ELx50-16"	BioTek	ELX5016	This plate washer contains a 16-channel manifold suitable for 384-well plates. It fits into a biosafety cabinet and has a lid covering the plate during washing which reduces the risk of aerosol production. Alternative plate washers with similar features could be used.
Gentamicin	Sigma-Aldrich	G1397	
Anhydrotetracycline hydrochloride	Sigma-Aldrich	37919	100 ug/ml solution in 100% ethanol is kept at -20°C protected from light in aluminum-foil
PBS	Gibco	20012	
Paraformaldehyde	Sigma-Aldrich	P6148	Dissolve in 0.2 M HEPES buffer, pH 7.4. Store at -20°C and thaw freshly the day before use. Caution, PFA is toxic by inhalation, in contact with skin and if swallowed.
HEPES	Sigma-Aldrich	H3375	
Triton X-100	Sigma-Aldrich	T9284	
DAPI	Roche	10236276001	

Scrambled siRNA	Dharmacon	D-001810-10	
Kif11 siRNA	Dharmacon	L-003317-00	
ARPC3 siRNA	Dharmacon	L-005284-00	
<i>Brucella abortus</i> 2308			
pJC43 (<i>apHT::GFP</i>)			Celli <i>et al.</i> ¹²
pAC042.08 (<i>apht::dsRed</i> , <i>tetO::tetR-GFP</i>)			Construction: pJC44 ⁴ was digested with EcoRI followed by generation of blunt ends with Klenov enzyme and subsequent digestion with SalI. TetR-GFP was amplified from pNF106 using primer prAC090 and prAC092. Following digestion with SalI, the TetR-GFP product was ligated to the digested pJC44 vector.
Primer prAC090	Sigma-Aldrich		TTTTTGAATTCTGGCA ATTCCGACGTCTAAGA AACC
Primer prAC092	Sigma-Aldrich		TTTTTGTCGACTTTGT CCTACTCAGGAGAGC GTTC
HeLa CCL-2	ATCC	CCL-2	

ImageXpress Micro	Molecular Devices	IXM IMAGING MSCOPE	Automated cellular imaging microscope equipped with a precision motorized Z-stage. Alternative systems for automated microscopy and alternative components for hard- and software specified below can be employed.
High-Speed Laser Auto-Focus	Molecular Devices	1-2300-1037	
CFI Super Fluor 10x objective	Nikon	MRF00100	N.A 0.50, W.D 1.20mm, DIC Prism: 10x, Spring loaded
Photometrics CoolSNAP HQ Monochrome CCD Camera	Molecular Devices	1-2300-1060	1392 x 1040 imaging pixels, 6.45 x 6.45- μ m pixels, 12 bits digitization
MetaXpress software	Molecular Devices	9500-0100	
LUI-Spectra-X-7	Lumencor	SPECTRA X V-XXX-YZ	Light engine. The following light sources are used: violet (DAPI), cyan (GFP), green/yellow (RFP)
Single Band Exciter for DAPI	Semrock	FF01-377/50-25	
Single Band Emitter for DAPI	Semrock	FF02-447/60-25	
Single Band Dichroic for DAPI	Semrock	FF409-Di03-25x36	
Single Band Exciter for GFP	Semrock	FF02-472/30-25	
Single Band Emitter for GFP	Semrock	FF01-520/35-25	

Single Band Dichroic for GFP	Semrock	FF495-Di03-25x36	
Single Band Exciter for RFP	Semrock	FF01-562/40-25	
Single Band Emitter for RFP	Semrock	FF01-624/40-25	
Single Band Dichroic for RFP	Semrock	FF593-Di03-25x36	



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Department: BIOZENTRUM
Institution: UNIVERSITY OF BASEL
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Editorial/reviewers comments and replies

Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. **Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word.**

2) Formatting

a) There are a few instances where portions of Steps are not in the imperative, and would be better-suited as notes. For example, "The pre-warmed...plate." in 2.11. Please check the manuscript for similar instances.

Notes were included in steps 2.1 and 2.11

b) Please check that abbreviations are defined at their first use (e.g., PFA in 3.10).

Abbreviations were defined for RNAi (introduction), siRNA (introduction), PFA (3.10), DAPI (4.3)

c) Please reformat the figure legends so that they are in paragraph form/all panels are discussed in the same paragraph.

Figure legends were formatted to paragraph form.

3) Visualization:

a) If 5.1 is to be filmed, additional step-wise detail must also be highlighted. However, it is recommended to remove the highlighting from 5.1.

Highlighting in section 5.1 was removed. However, we believe that adding an illustrative short sequence showing that the plates are imaged on an automated microscope might benefit the flow of the story told in the video sequence.

b) Prior to peer review, the highlighted portion of your protocol is our 2.75 page highlighting limit (about 3 pages are highlighted). Please adjust the highlighting to identify a total of no more than 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. Finally, please ensure that the total length of the protocol is within 10 pages.

The protocol is within the 10 page limits and the number of highlighted sections is within 2.75 pages.

4) Unnecessary branding should be removed:

a) 5.1 Note - Indicate that other microscopes/software can be used. If this caveat is not included, MetaXpress and Molecule Device ImageXpress references will need to be removed.

The following statement was included “Alternative 2D widefield microscopy devices with appropriate hardware specifications and imaging software can be used (see Table of Material/Equipment for details).”

b) 6 Note - Although CellProfiler is open source, it is over-used in this note. Please reduce the number of times this software is mentioned to two.

The term “Cell Profiler” is now used two times in this note.

5) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

6) Please disregard the comment below if all of your figures are original.

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No figures were re-used.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a high throughput technique for the identification of non-redundant factors required for the success of entry/early survival or proliferation in HeLa cells. Moreover, the authors present a system that could distinguish extracellular vs. intracellular bacteria, which is particularly critical when a high multiplicity of infection is used. I found the manuscript interesting and clear, and I have only a few minor suggestions.

Major Concerns:

None

Minor Concerns:

*Clarity of presentation:

The description is very clear. I have a few minor details:

- What is the "provided pipeline" (between sections 6 and 6.1) ? It could be interesting to describe this with one or two sentences.

The term "pipeline" was describe by the addition of the following statement. "The software provides image analysis algorithms in individual modules, which can be combined into a pipeline that will execute the modules consecutively on all images, to automatically perform a specific image analysis task."

- In the system used, is there a unique choice for the camera? If not, maybe it could be interesting to specify which camera was mounted, because form parameters are given in pixels

We specified the microscope components including the camera in the materials list.

ImageXpress Micro	Molecular Devices	IXM IMAGING MSCOPE	Automated cellular imaging microscope equipped with a precision motorized Z-stage. Alternative systems for automated microscopy and alternative components for hard- and software specified below can be employed.
High-Speed Laser Auto-Focus	Molecular Devices	1-2300-1037	
CFI Super Fluor 10x objective	Nikon	MRF00100	N.A 0.50, W.D 1.20mm, DIC Prism: 10x, Spring loaded

Photometrics CoolSNAP HQ Monochrome CCD Camera	Molecular Devices	1-2300-1060	1392 x 1040 imaging pixels, 6.45 x 6.45- μ m pixels, 12 bits digitization
MetaXpress software	Molecular Devices	9500-0100	
LUI-Spectra-X-7	Lumencor	SPECTRA X V-XXX-YZ	Light engine. The following light sources are used: violet (DAPI), cyan (GFP), green/yellow (RFP)
Single Band Exciter for DAPI	Semrock	FF01-377/50-25	
Single Band Emitter for DAPI	Semrock	FF02-447/60-25	
Single Band Dichroic for DAPI	Semrock	FF409-Di03-25x36	
Single Band Exciter for GFP	Semrock	FF02-472/30-25	
Single Band Emitter for GFP	Semrock	FF01-520/35-25	
Single Band Dichroic for GFP	Semrock	FF495-Di03-25x36	
Single Band Exciter for RFP	Semrock	FF01-562/40-25	
Single Band Emitter for RFP	Semrock	FF01-624/40-25	
Single Band Dichroic for RFP	Semrock	FF593-Di03-25x36	

- Section 6.2.2.3.13) and 14) : what do the authors means by "child" and "children"? Not clear to me.

The terms are now explained in the supplemental code file by the following statement: “We will call this relation a parent-child-relation, where the *Brucella* colonies that relate to a Voronoi cell body are the “children” of this Voronoi cell body.”

*Scientific accuracy:

The scientific accuracy is excellent.

*Compliance with research standards:

This work matches the expected standards in the field. I would just suggest to the authors to propose to the user to check that their *Brucella* strain is Smooth and not Rough before starting such a huge experiment.

We agree with the reviewer that knowing the state of the LPS is crucial. We thus added the following note in 1.1.1: “Note: The smooth LPS of *Brucella abortus* and other *Brucella* strain is an important virulence determinant but rough mutants can occur at relatively high frequencies¹⁴. We thus recommend testing the status of the culture strain before starting with this experiment.”

*Technical quality and efficiency:

The article describes an experiment with a high technical quality. The concentration of kanamycin is a little bit high, but it should not impact the procedure if the genotype is stable.

50 ug/ml of kanamycin has been extensively used in the field and by the authors of the plasmid (Celli et al., 2004, PMID: 15632218) and should not have an impact. In fact, we have experienced that lower concentrations of kanamycin can affect the stability of the plasmid and may lead to the loss of the plasmid.

*General impact and usefulness:

Low because equipment and associated costs are only accessible to a very limited number of laboratories worldwide. However, the easy distinction between intracellular and extracellular bacteria is interesting and can be applied in any lab having a BSL3 and able to genetically manipulate *Brucella abortus*.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This is a superb report which describes methods for performing a screen for host factors that support the intracellular replication and/or entry of *Brucella* in host cells. The report will be of broad interest to the intracellular bacterial pathogenesis research community.

Major Concerns:

None

Minor Concerns:

The inactivation or knockdown of some host factors, especially those that are essential for cell viability, is expected to result in cell death. Such hits run the risk of generating false positives in the screen.

The narrative would benefit from an explicit explanation of how the quantification scheme addresses this issue.

We agree with the reviewer that the interpretation of the results is a critical part of RNAi screening. Besides the issue of cell number effects, also siRNA driven off-target effects, knockdowns efficiency, target expression, etc. need to be considered. Unfortunately, a detailed discussion of these aspects goes beyond the scope of this manuscript.

Since the cell number phenomenon directly impacts infection scoring discussed in section 7 we added the following note: "Note: siRNAs which have a significant impact on cell viability have to be considered with caution, since this can promote false positive discoveries. An altered cell number affects the actual MOI and targeting of essential genes can have pleiotropic effects on pathogen infection. While the incomplete depletion by siRNAs allows for the study of essential genes, such targets have to be validated by alternative methods (e.g. pharmaceutical interference) to corroborate their role as host factors during infection."

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

The manuscript "HT microscopy-based RNA interference assays to study host factors involved in Brucella infection of HeLa cells" provides experimental protocols for the measurement of two events during Brucella invasion, first bacterial intracellular proliferation, and second bacterial entry. Overall, the provided protocols are detailed and allow their reproduction by other scientists. The ms is generally well written and comprehensible. Also, the provided data illustrates well the kind of information one can obtain with the two assays.

Major Concerns:

no major concerns.

Minor Concerns:

I would like to mention a couple of issues that would improve the manuscript further. Taking them into account would be appreciated.

1) The title could be misleading- as discussed by the authors, the assay works in conjunction with siRNA screens. However, this is only one of its applications. Therefore, this could be rephrased. For example "Microscopy-based assays for HT screening to study *Brucella* infection of HeLa cells". The siRNA screening issue (in the end, no screen is provided in this manuscript) comes up several times throughout the manuscript. Again, I suggest to rephrase and highlight that it can be used for such screens, but the protocols themselves are also useful outside the siRNA screening context.

We altered the title to the following: "Microscopy-based assays for high-throughput screening of host factors involved in *Brucella* infection of HeLa cells"

In order to account for the changes and indicate that alternative screening technologies could be used in conjunction with the method, we included the following statement in the long abstract: "The protocols describe the use of RNA interference, while alternative screening methods could be applied."

2) The short abstract is only fully understandable after reading the whole manuscript, it may be good to rephrase it.

We rephrase the short abstract to reduce its complexity.

3) Important: The "entry assay" is not well introduced (but well discussed at the end of the ms). It would be good to point out its limitations- for example what is the shortest entry time that can be followed with the approach. Also, the usage of two colors (GFP and dsRed) is very nicely discussed from line 657 onwards. It would have been great to have this part substantiated in figure 2 showing the two fluorophors.

We described the entry assay in more detail at the end of the introduction.

We commented on the raised issue concerning the shortest possible entry time by adding the following sentences: "In order to reach detectable GFP expression by intracellular *Brucella* we have found that 4 h of induction by aTc results in a reliable signal."

We adjusted figure 2 accordingly. It now shows both bacterial signals, GFP and dsRed. The figure legend was changed accordingly.

4) Can the 384 well format be a limitation? Does it provide enough cells for robust biological interpretations in case the differences are not as strong as with the siRNAs presented in the ms?

At the end of the assay the number of HeLa cells reaches ~2000 for the entry assay and ~4000 for the endpoint assay. We thus do not consider the format a limiting factor, while it is clear that certain mild effects might not be captured.

5) The voronoi cell bodies should be defined in the text. In fact, I was surprised by their shape here.

The Voronoi cell body was explained in the text the first time it appeared with the following note under 6.1.2.10: "Note: The Voronoi cell body is a radial extension of the nucleus by 25 pixels with no overlap with neighboring voronoi cell bodies."

In addition, the Voronoi cell body and peri nucleus were described more precisely in figure legend 1A.

6) Important: The description of the image analysis section needs to be presented not as a "simple-to-follow" protocol, but the general principles should be briefly brought up including issues that could arise. This would help scientists that use other imaging systems to work with the assays and to continue with cell profiler. For example, the value "0.02" (line 404) comes somewhat out of the blue. I think, it's better to mention a few general issues first, such the effect of imaging at different resolution, different pixel sizes, etc..., and then portray the analysis pipeline.

[Editorial recommendation: Please keep JoVE's protocol requirements in mind as you address the above comment and similar comments- the protocol should be composed of short steps written in the imperative tense (directing the user to do something). Please avoid large paragraph style non-imperative tense text in the protocol. An in-depth discussion of protocol steps (e.g. rationale behind steps, modifications etc.) may be included in the Discussion.]

An in depth discussion of all image analysis relevant principles goes beyond the scope of this article. However, we agree that depending on the imaging setting that are used (especially of the dynamic range is not optimally chosen) alternative parameter than the values indicated might be optimal. We thus changed the following statements in the protocol to account for these differences without compromising the flow of the protocol:

6.1.2.7: we suggest starting with the value zero instead of 0.04

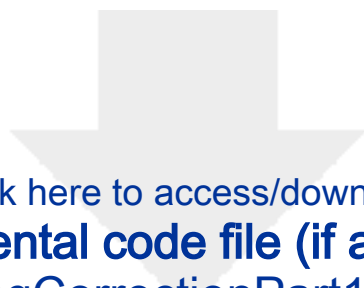
6.1.2.8.1: we suggest starting with the value zero instead of 0.02

7) Important: I am not sure whether a 20 min PFA treatment is generally accepted to take Brucella out of the BSL3 environment. It depends on the specific security protocols in place in different research institutions. Since the ms portrays a protocol, this should be mentioned clearly.

We agree with the reviewer and added to following statement to warn the user: “Caution: Removal of any samples from a BSL3 facility is subject to risk assessment and validation of the procedure and depends on the applicable regulations for biosafety.”

Additional Comments to Authors:

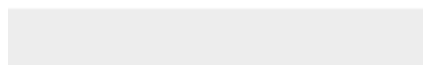
no additional comments.



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Supplemental code file (if applicable)

BrucellaShadingCorrectionPart1-Ver007.cppipe





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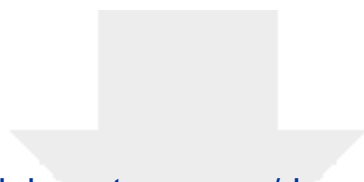
BrucellaEndpointWithShadingCorrectionPart2-
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