**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 annually[1](#_ENREF_1). 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 neuropathy[2](#_ENREF_2).

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 Cdc42[3](#_ENREF_3). Inside the eukaryotic host cell, the BCV traffics along the endocytic pathway and despite the interaction with lysosomes, bacteria manage to avoid degradation[4](#_ENREF_4). Acidification of the BCV by the vesicular ATPase is required to induce the expression of the bacterial type IV secretion system (T4SS)[5](#_ENREF_5). It is believed that bacterial effectors secreted by the T4SS are essential for *Brucella* to establish its replicative niche, since deletion of the T4SS[6](#_ENREF_6) or inhibition of the vesicular ATPase lead to defects in the establishment of the intracellular niche[7](#_ENREF_7). Bacteria do not replicate during the phase of trafficking until theyreach an ER-derived vacuolar compartment[8](#_ENREF_8). Once intracellular proliferation occurs, the BCV is found in close association with ER markers such as calnexin and glucose-6-phosphatase[6](#_ENREF_6).

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* cells[9](#_ENREF_9). 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](#_ENREF_10),[11](#_ENREF_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](#_ENREF_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](#_ENREF_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 frequencies[14](#_ENREF_14). 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 2[15](#_ENREF_15) 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.

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 cells[3](#_ENREF_3). 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 μm). 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 μm. **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. abortu*s showing dsRed and GFP signal, red = extracellular *B. abortus* showing dsRed signal, blue = nuclei stained with DAPI, scale bar = 50 μm). *B. abortus* expressing dsRed andTetR-GFPwere 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 ofinfection 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 pathogens[10](#_ENREF_10). 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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