**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.

If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

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-µm 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 frequencies[14](#_ENREF_14). We thus recommend testing the status of the culture strain before starting with this experiment.”  
  
\*Technical quality and efficiency:  
  
The articles describes an experiment with a high technical quality. The concentration of kanamycine is 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 only accessible to a very limited number of laboratory 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 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.