Video Article

A Microscopic Phenotypic Assay for the Quantification of Intracellular Mycobacteria Adapted for High-throughput/High-content Screening

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Abstract

Despite the availability of therapy and vaccine, tuberculosis (TB) remains one of the most deadly and widespread bacterial infections in the world. Since several decades, the sudden burst of multi- and extensively-drug resistant strains is a serious threat for the control of tuberculosis. Therefore, it is essential to identify new targets and pathways critical for the causative agent of the tuberculosis, *Mycobacterium tuberculosis* (*Mtb*) and to search for novel chemicals that could become TB drugs. One approach is to set up methods suitable for the genetic and chemical screens of large scale libraries enabling the search of a needle in a haystack. To this end, we developed a phenotypic assay relying on the detection of fluorescently labeled *Mtb* within fluorescently labeled host cells using automated confocal microscopy. This *in vitro* assay allows an image based quantification of the colonization process of *Mtb* into the host and was optimized for the 384-well microplate format, which is proper for screens of siRNA-, chemical compound- or *Mtb* mutant-libraries. The images are then processed for multiparametric analysis, which provides read out inferring on the pathogenesis of *Mtb* within host cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/51114/

Introduction

Among the emerging and re-emerging infectious pathogens reported during the last years, *Mycobacterium tuberculosis* (*Mtb*) holds a prominent place being responsible for 1.4 million deaths and 8.7 million new infections in 2011 (Global tuberculosis report 2012, www.who.int/topics/tuberculosis/en/). Despite the availability of multidrug therapies, the number of infected people is still on the rise and multidrug resistant (MDR) as well as extensively drug resistant (XDR) *Mtb* are quickly spreading all over the world[1]. Moreover, when taking into consideration the presence of *Mtb* antigens, it is evident that one third of the global population is considered as being latently infected by Mtb. Statistically, in one case out of ten, there is evolution towards the active form of the disease with subsequent clinical symptoms[2]. Therefore, new means to fight *Mtb* are urgently needed. In this context, we developed an *in vitro* visual phenotypic assay relying on monitoring *Mtb* invasion and multiplication into host cells by automated confocal fluorescence microscopy[3]. The adaptation of the assay in 384-well microtiter plates in combination with automated image acquisition and analysis, allowed High-content/High-throughput Screening (HC/HTS) of medium scale libraries of compounds, siRNAs and bacterial mutants. The screening of a genome wide RNAi library on this phenotypic assay thus enabled the identification of the key host-factors involved in *Mtb* trafficking and intracellular replication but also the elucidation of host-pathways exploited by the tubercle bacillus. Another adaptation of this particular phenotypic assay was for the identification of bacterial factors essential to *Mtb* intra-phagosomal persistence. For instance, the arrest of phagosome maturation is considered as one of the major mechanisms that facilitates the survival and replication of *Mtb* in macrophage. The monitoring of the subcellular localization of *Mtb* knock-out mutants in fluorescently labeled-acidic compartments allowed for the identification of bacterial genes involved in the survival process[4]. Finally, the high-content imaging of *Mtb* also offers an excellent method to quantify drug efficiency for inhibiting various phenomena like intracellular bacterial growth[3]. Altogether, this type of high throughput phenotypic assay allows accelerating drug discovery against TB and the data collected by these different approaches contribute to a better understanding of the host manipulation exerted by *Mtb*.

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Protocol

1. High-throughput Genome-wide siRNA Screening

Screening performed in a human Type-II pneumocytes model A549 cell line upon infection with Mtb H37Rv expressing Green Fluorescent Protein (GFP). This procedure is outlined in **Figure 1A**.

1. Resuspend the dried siRNA library that is stored in mother plates (96-well plates) with 1x siRNA buffer to reach a concentration of 4 µM, then transfer 10 µl of the mixture into a 384-well daughter plate (daughter plate 1).
2. Add 10 µl of 1x siRNA buffer in daughter plate 1 to dilute siRNA by 2-fold. After siRNA resuspension, plates are sealed with peelable aluminum seal and can be stored at -20 °C at least 6 months and up to 2-3 years, but storage time may vary depending on siRNA library manufacturer's recommendations.
3. Dilute siRNAs in daughter plate 1 into daughter plate 2 to reach a concentration of 500 nM. After siRNA resuspension, plates are sealed with peelable aluminum seal and can be stored at -20 °C at least 6 months and up to 2-3 years, but storage time may vary depending on siRNA library manufacturer's recommendations.
4. Before use, thaw daughter plate 2 at room temperature.
5. Take 2.5 µl of siRNA from daughter plate 2 and place into a 384-well assay plate.
6. In the same 384-well assay plate in step 1.5, add 2.5 µl negative and positive control siRNA to their respective wells.
7. Dilute the transfection reagent in 1x D-PBS to yield enough solution to provide 0.1 µl transfection reagent in each well and preincubate the diluted transfection solution at room temperature for 5 min.
8. Add 7.5 µl of the transfection reagent/PBS solution mixture to each well in the assay plate and incubate for 30 min at room temperature.
9. Add 40 µl of A549 cells (1,500 cells/well) suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Maintain cells for a 3 day-incubation period at 37 °C in an atmosphere containing 5% CO_2. These cells divide every 24 hr, thus about 12,000 cells are in the wells three days after transfection.
10. Wash a two-week old GFP-expressing *Mtb* H37Rv culture out with D-PBS (free from $MgCl_2$ and $CaCl_2$) by centrifugation at 4,000 x g for 5 min and discard the washes. Repeat this step 3x. (For GFP-*Mtb* H37Rv culture conditions see Protocol 3).
11. Suspend the bacterial pellet in 10 ml of RPMI 1640 medium supplemented with 10% FBS and decant for 1 hr at room temperature to allow bacterial aggregates to sediment.
12. Collect the bacterial supernatant and measure OD_{600} (OD_{600} should be between 0.6-0.8) and GFP-fluorescence (RFU value) using a microplate reader to determine the bacterial concentration. Calculate the titer of the suspension using a reference regression line displaying RFU value = f (CFU value) that had been generated prior to the experiment on another culture that had been prepared in the same conditions. Prepare bacterial suspension containing 2.4×10^6 bacteria/ml, which corresponds to a multiplicity of infection (MOI) of 5.
13. Remove the medium in the 384-well assay plate and add 25 µl of freshly prepared bacterial suspension.
14. Incubate the 384-well assay plate at 37 °C for 5 hr in an atmosphere containing 5% CO_2.
15. Remove the medium and gently wash the cells with RPMI medium supplemented with 10% FBS 3x.
16. To kill the remaining extracellular bacteria, treat the cells with 50 µl of fresh RPMI-FBS medium containing 50 µg/ml of amikacin at 37 °C for 1 hr in an atmosphere containing 5% CO_2.
17. Remove the medium-containing amikacin and add 50 µl of fresh RPMI medium supplemented with 10% FBS. Incubate the 384-well assay plate at 37 °C for 5 days in an atmosphere containing 5% CO_2.
18. Prior to image acquisition, add 10 µl of freshly prepared 30 µg/ml of DAPI in PBS (final concentration 5 µg/ml) and incubate for 10 min at 37 °C.
19. Load the plate into automated confocal microscope.

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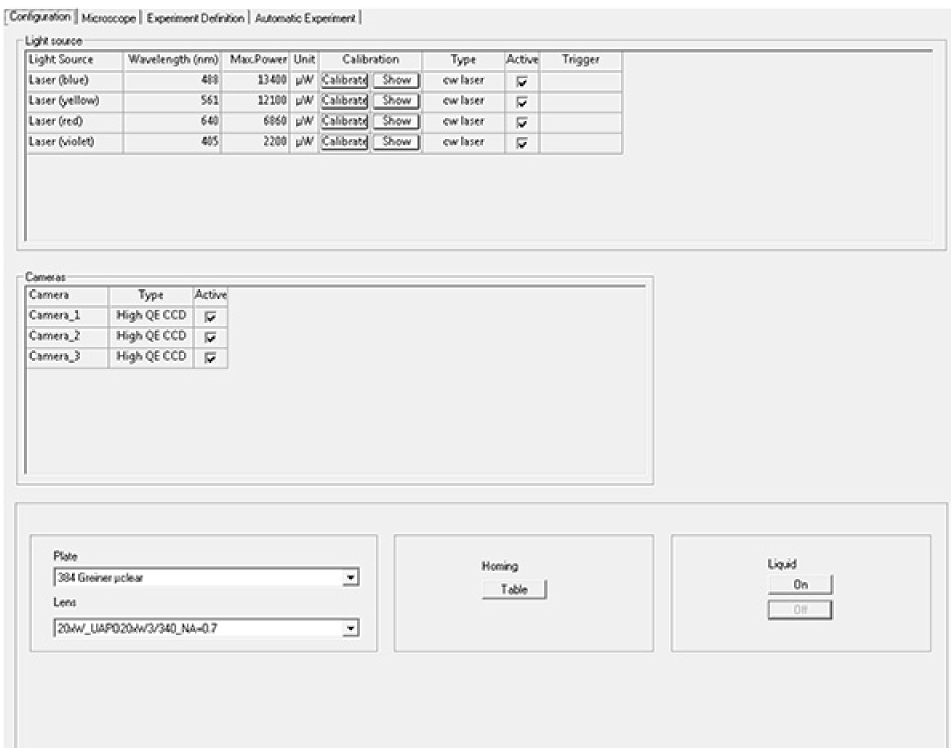

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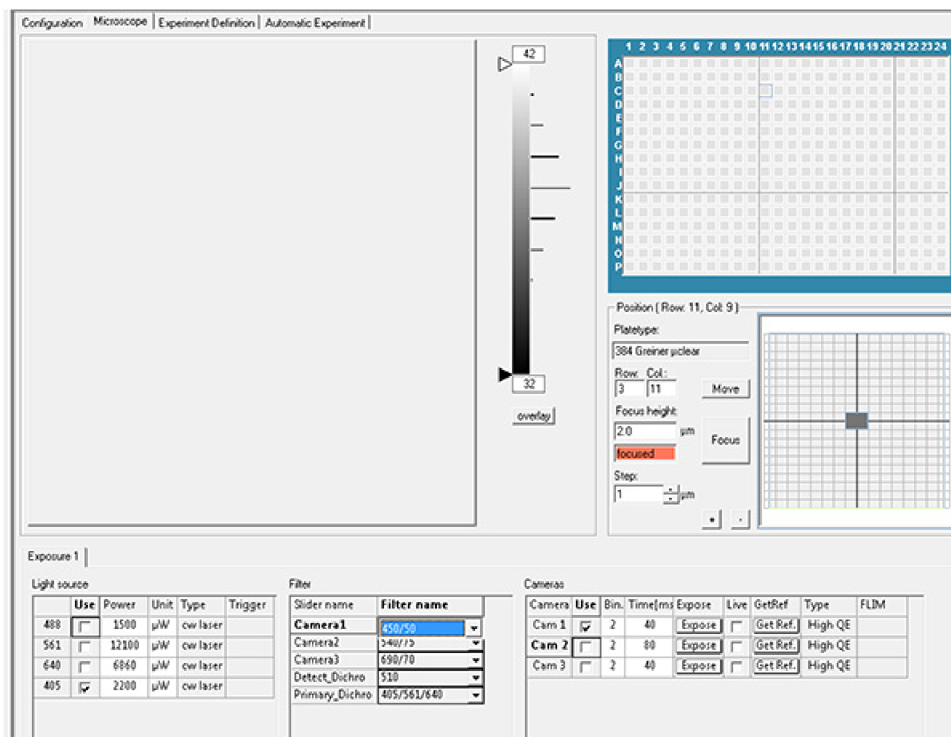
20. Set the exposure parameters. Record DAPI fluorescence using excitation laser 405 nm with emission filter 450 nm and GFP fluorescence using excitation laser 488 nm with emission filter 520 nm.



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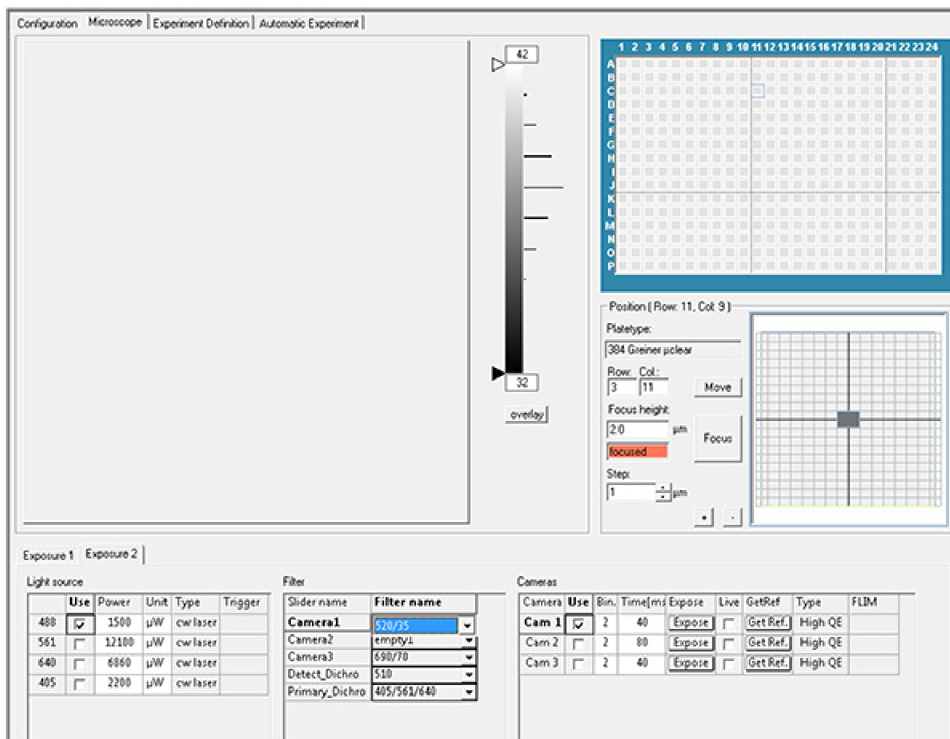

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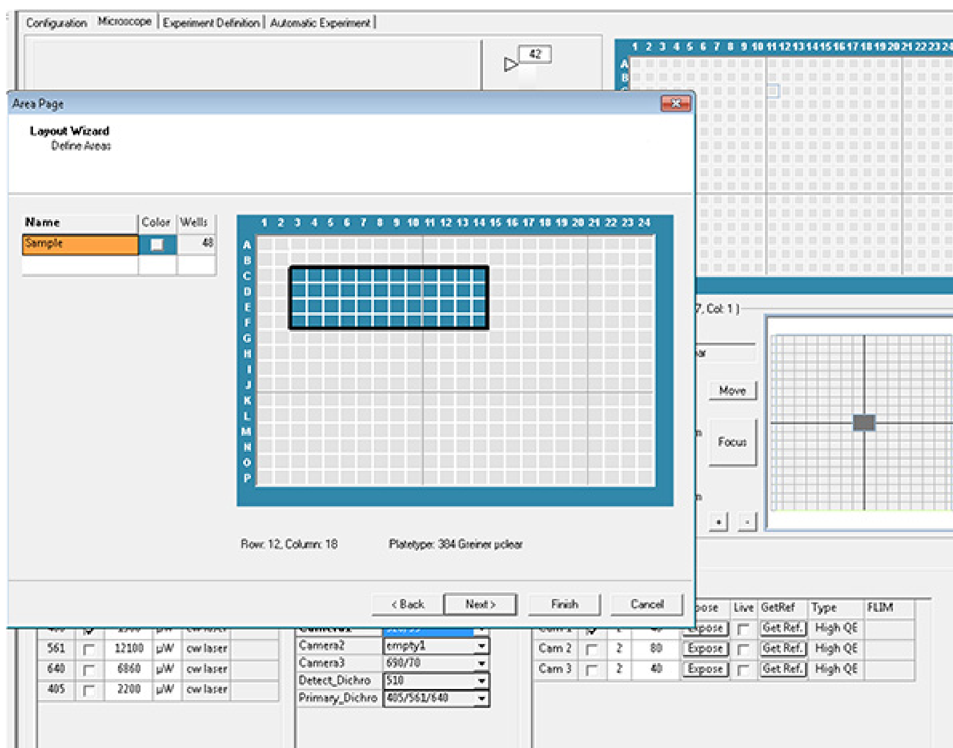
21. Select the wells and the fields in each well to be acquired, which is then referred as layout and sublayout parameters.



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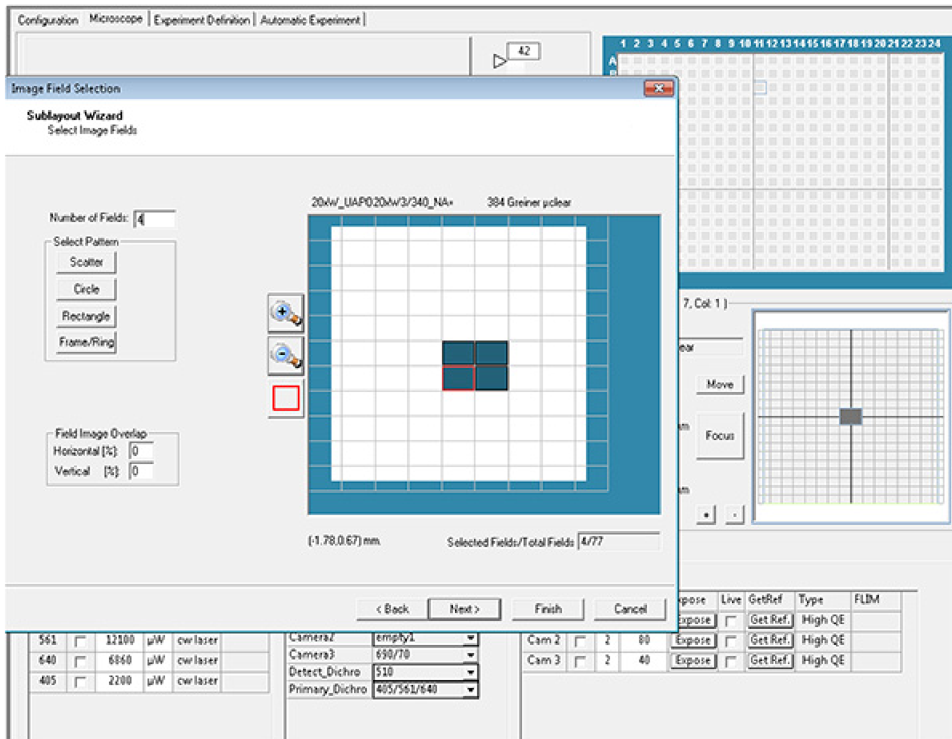

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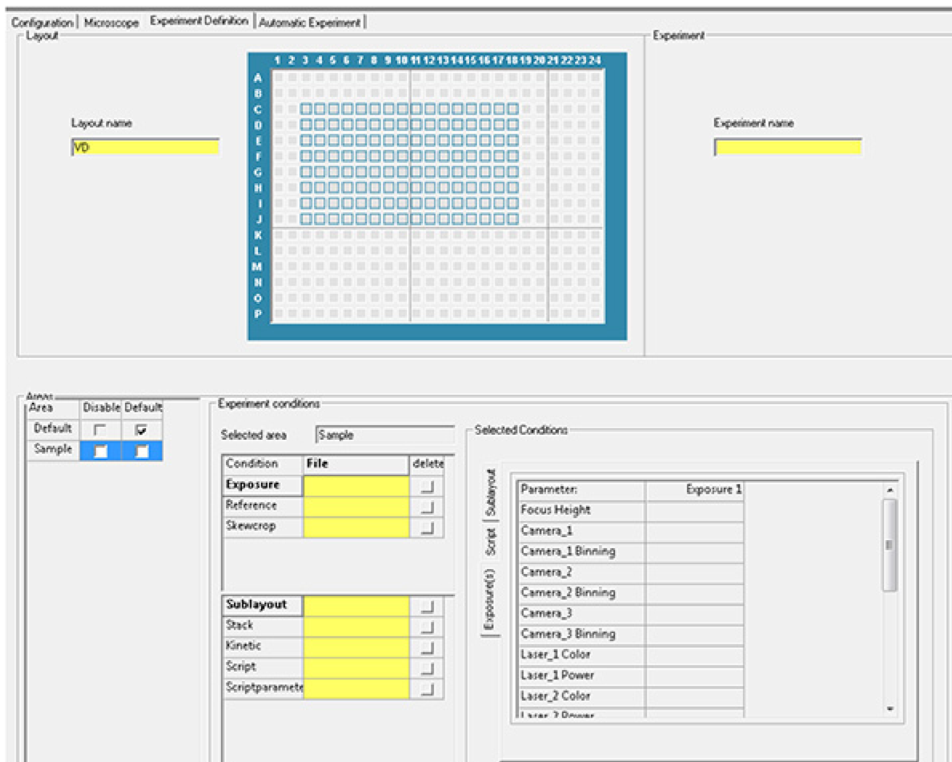
22. Generate the experiment file using the parameters from steps 1.20 and 1.21, and run the automatic acquisition.



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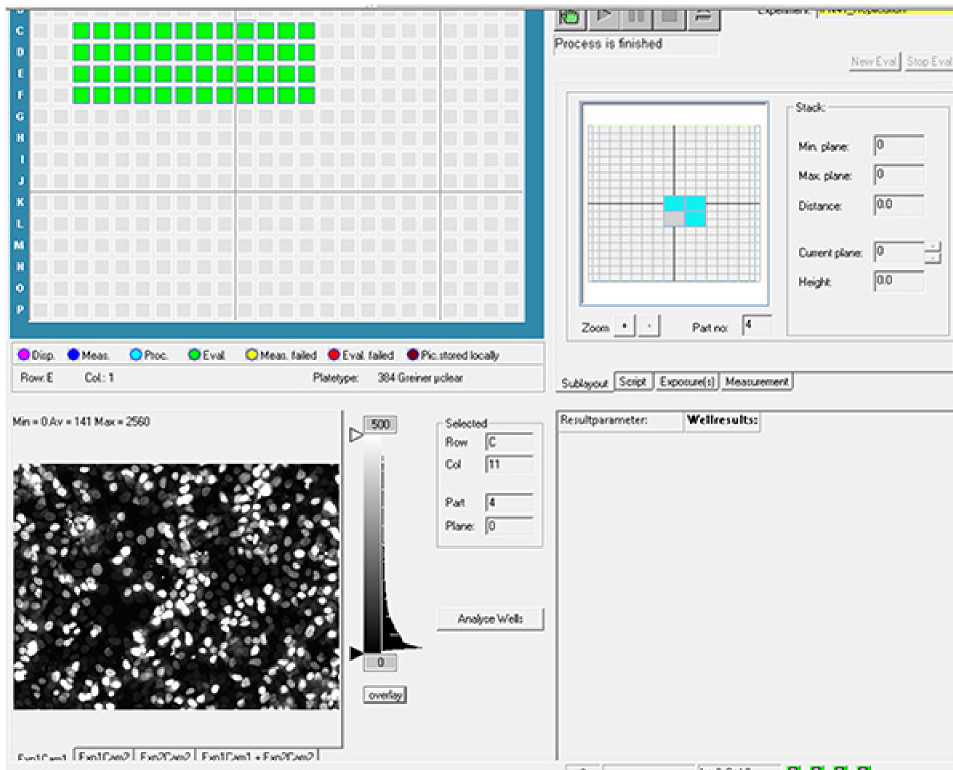

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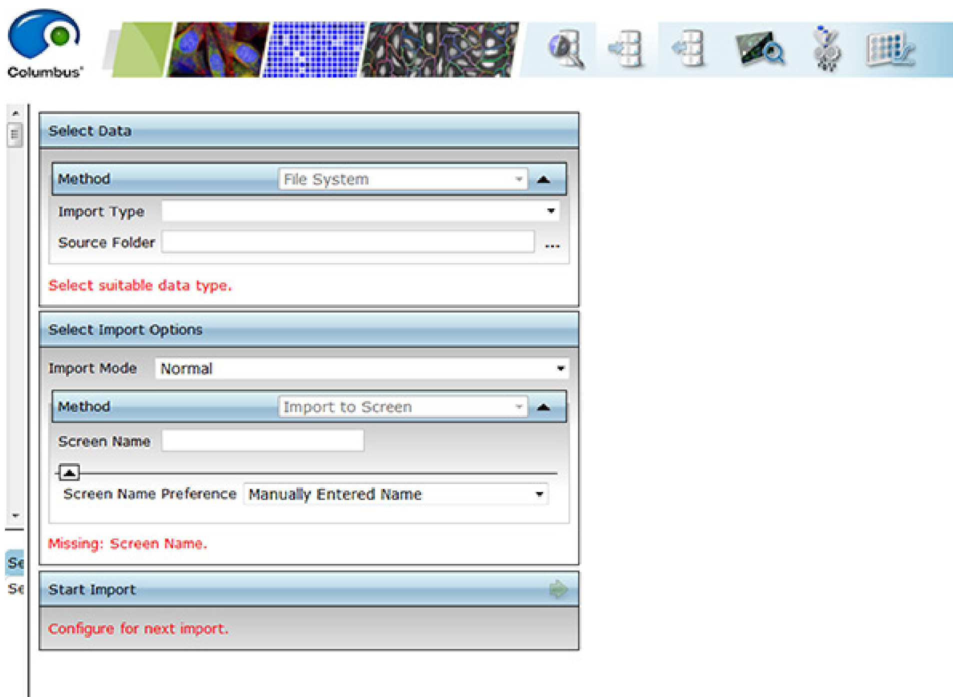
23. Transfer images to remote server.



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24. Evaluate images using image analysis software. Detect cell nuclei from DAPI channel using nuclei detection algorithm and the bacterial area from GFP channel using pixel intensity properties algorithm (**Figure 4A**).

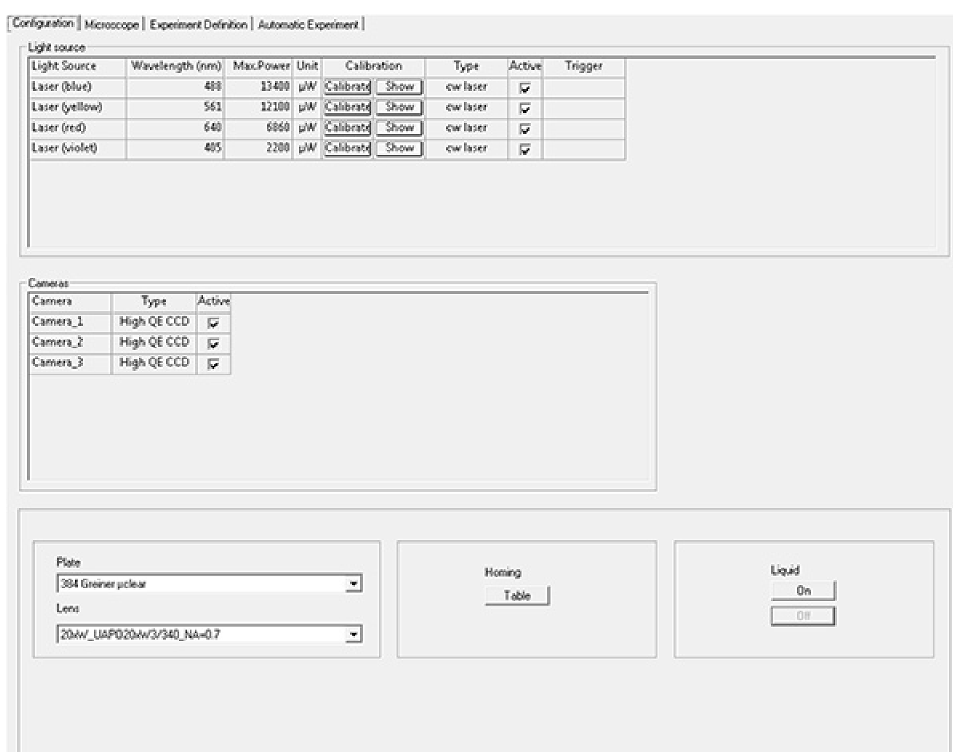
Note: This protocol is optimized to study the effect of gene silencing on the intracellular *Mtb* growth. *Mtb* is a slow-growth bacterium which divides every 20 hr in optimal conditions. After 5 days post-infection the amount of extracellular *Mtb* is still low in absence of cell lysis and didn't affect

the quality of the analysis. This protocol must be optimized in terms of length of antibiotic treatment and incubation time to be adapted for siRNA screens using fast-growth bacteria like *Mycobacteria smegmatis* and *Escherichia coli* that are extensively released and can infect new cells.

2. High-throughput Compound Screening

Screening performed on Mtb H37Rv infected host cells. This procedure is outlined in **Figure 1B**.

1. Thaw the 384-well mother plates containing the compound library solubilized in DMSO 100%. Transfer 0.5 µl of the compounds in 384-well daughter plates containing 10 µl of RPMI 1640 medium supplemented with 10% FBS.
2. Wash a two-week old GFP-expressing *Mtb* H37Rv culture out with D-PBS (free from $MgCl_2$ and $CaCl_2$) by centrifugation at 4,000 x g for 5 min and discard the washes. Repeat this step 3x (For GFP-*Mtb* H37Rv culture conditions see Protocol 3).
3. Suspend the bacterial pellet in 10 ml of RPMI 1640 medium supplemented with 10% FBS and decant for 1 hr at room temperature to allow bacterial aggregates to sediment.
4. Collect the bacterial supernatant and measure OD_{600} (OD_{600} should be between 0.6-0.8) and GFP-fluorescence (RFU value) using a microplate reader. Calculate the titer of the suspension using a reference regression line displaying RFU value = f (CFU value) that had been generated prior to the experiment. Typical concentration is 1 x 10^8 bacteria/ml.
5. Harvest 6 day old primary human macrophages at 4 x 10^5 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 ng/ml recombinant human M-CSF (For human Peripheral Blood Monocyte Cells purification and macrophages differentiation see Protocol 4).
6. Incubate the diluted primary cells with bacilli at different MOI, ranging from 1-5, in suspension with mild shaking at 90 rpm for 2 hr at 37 °C.
7. Wash the infected cells by centrifugation at 350 x g to remove the extracellular bacteria. After each centrifugation step, resuspend the pellet in RPMI 1640 medium supplemented with 10% FBS. Repeat this step 2x.
8. Suspend the infected cells in RPMI 1640 medium supplemented with 10% FBS and amikacin at 50 µg/ml and incubate the suspension with mild shaking for 1 hr at 37 °C.
9. Remove the cell-culture medium containing amikacin by centrifugation at 350 x g and wash the infected cells with complete RPMI 1640 medium supplemented with 10% FBS and 50 ng/ml recombinant human M-CSF. Repeat once.
10. Add 40 µl of the infected macrophages suspension in the same assay plate in step 2.1, which already contains 10 µl of the compound dilutions. The final concentration of DMSO in each well has now reached 1%.
11. Incubate the assay plates for 5 days at 37 °C in an atmosphere containing 5% CO_2.
12. Stain the live cells with cell-permeant far-red fluorescent dye.
13. Load the plate into automated confocal microscope.



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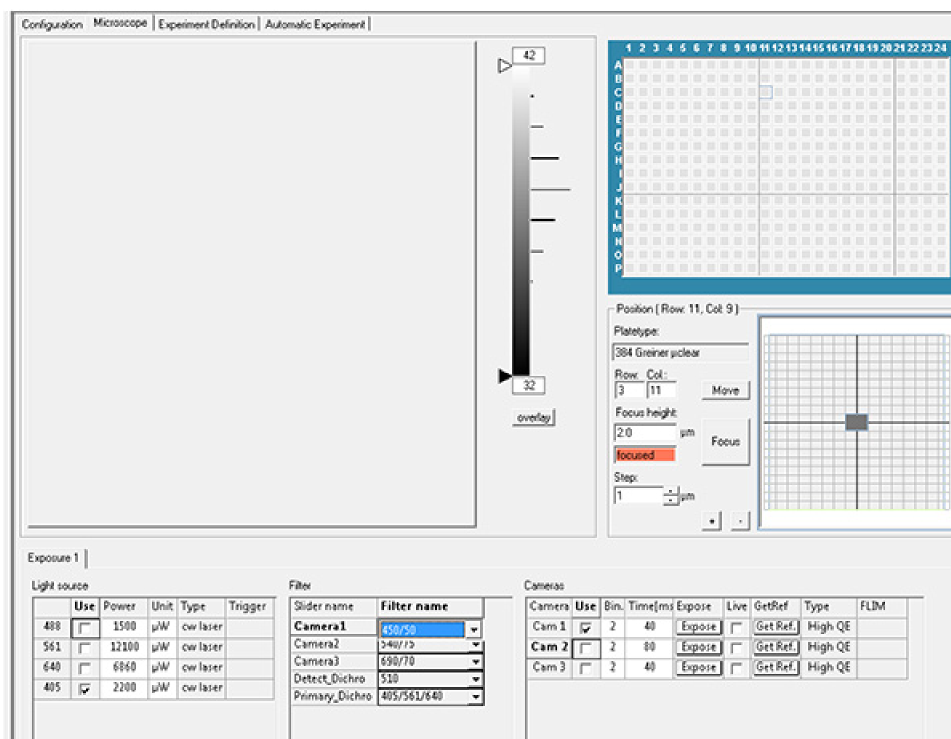
14. Set the exposure parameters. Record far-red fluorescence using excitation laser 640 nm with emission filter 690 nm and GFP fluorescence using excitation laser 488 nm with emission filter 520 nm.



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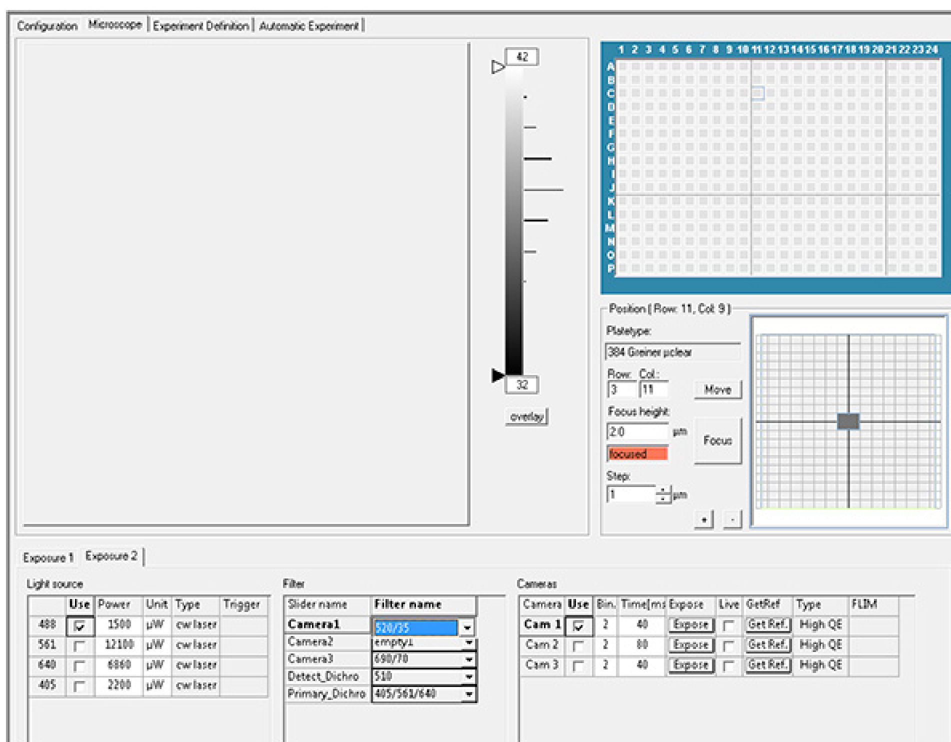

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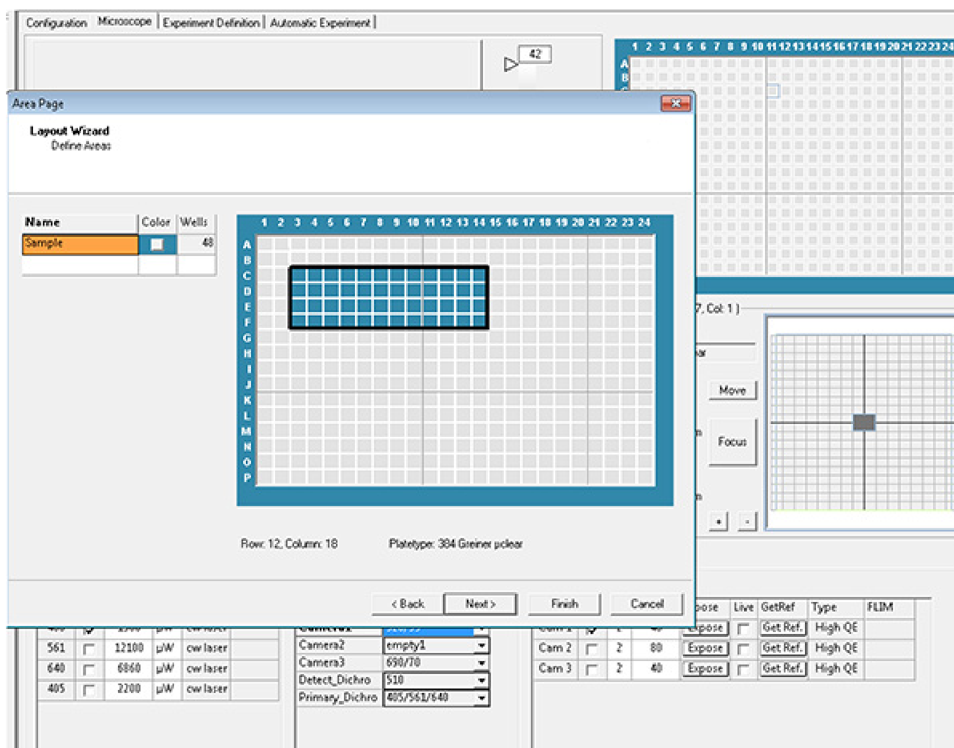
15. Select the wells and the fields in each well to be acquired, which is then referred as layout and sublayout parameters.



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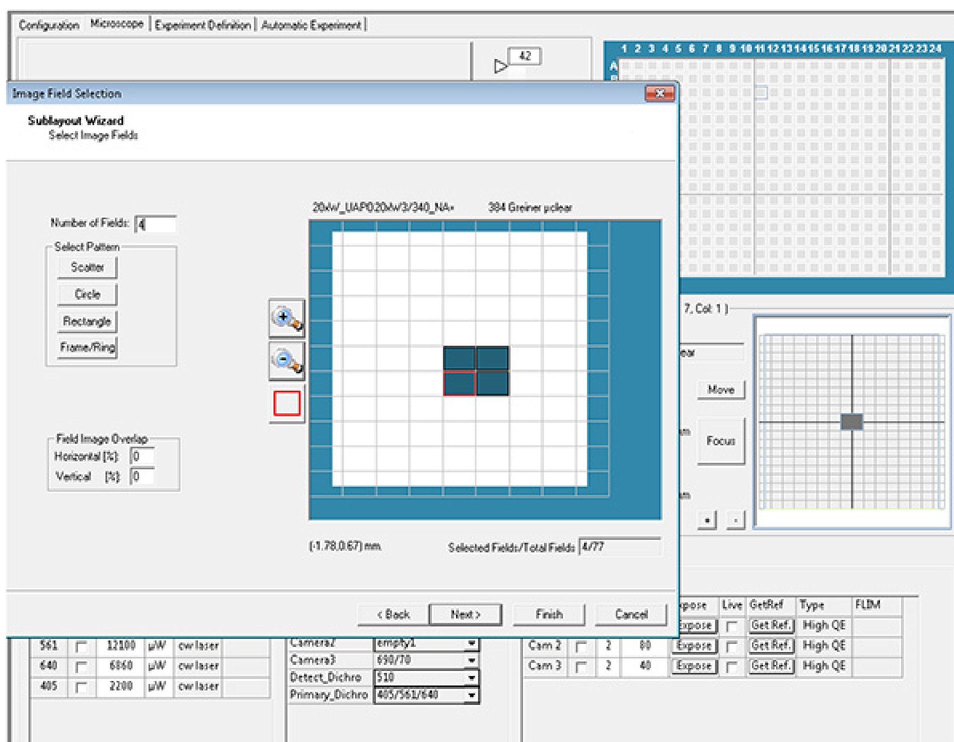

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16. Generate the experiment file using the parameters from steps 2.14 and 2.15 and run the automatic acquisition.

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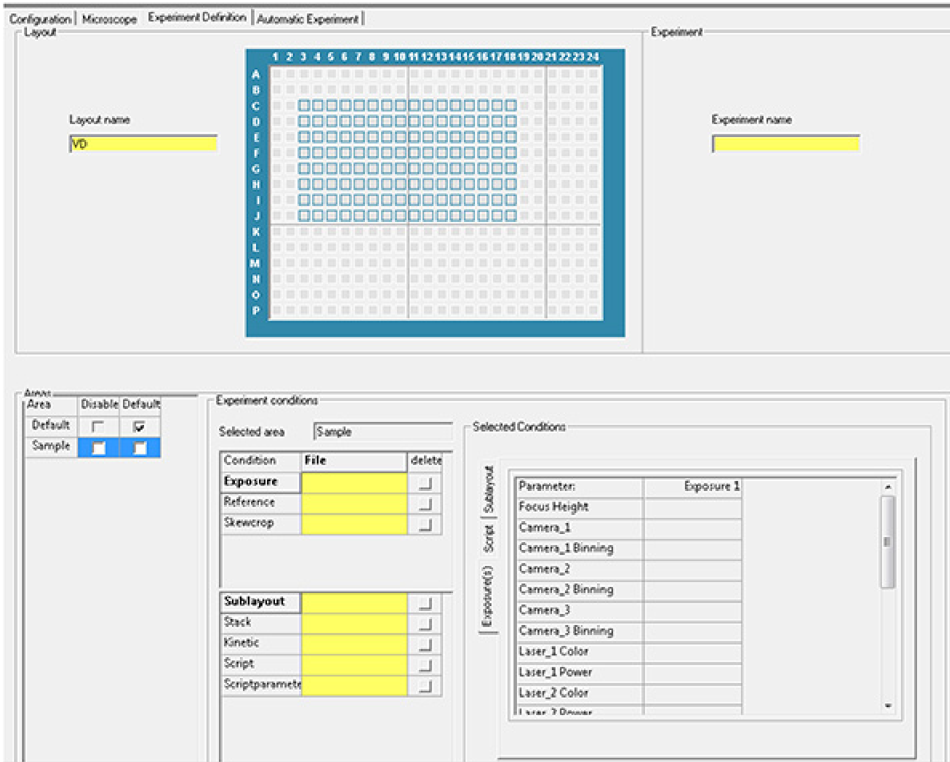

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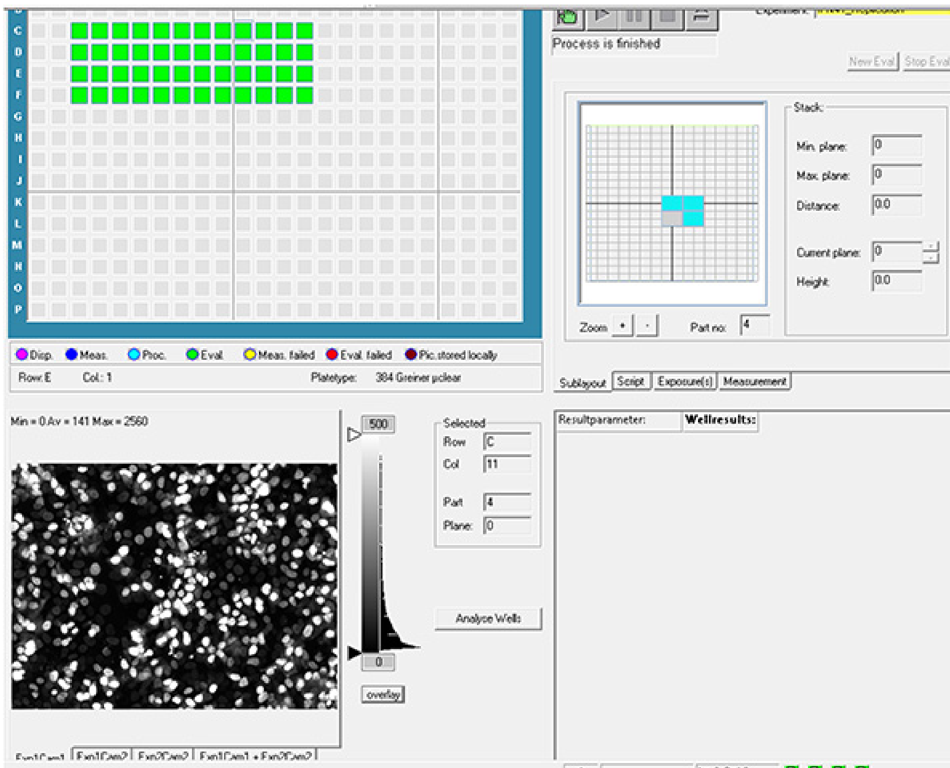

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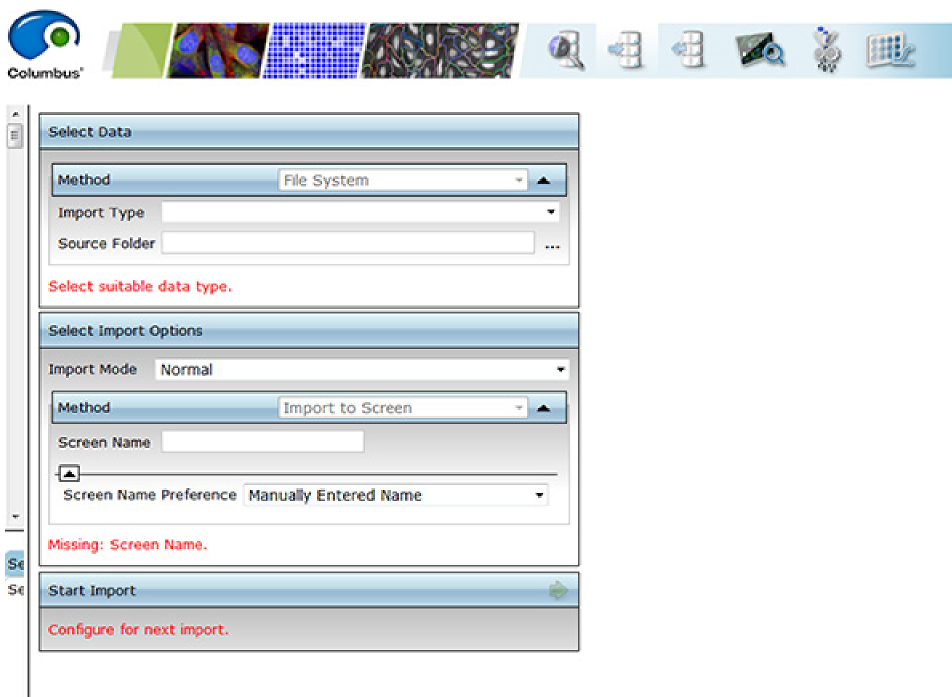
17. Transfer images to remote server.



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18. Evaluate images using image analysis software. Detect cell area from far-red channel using pixel intensity properties algorithm and bacterial area from GFP channel using pixel intensity properties algorithm (**Figure 4B**).

Note: This protocol can be adapted for *Mtb* mutant library screening by replacing compounds by mutants expressing a fluorescent protein (One well/One mutant) (**Figure 1C**, see also Brodin *et al.*[4]). Fluorescent mutants are first seeded in wells (20 µl of bacterial suspension per well). Bacteria are then recovered by 30 µl of cell suspension. After centrifugation at 350 x g for 1 min, the plate is incubated at 37 °C in an atmosphere containing 5% CO_2. Incubation time and MOI depend on the assay. As an example, for visualization of early cellular events such as phagosome acidification, the cells can be infected for 2 hr with MOI ranging from 1-20. Lysosomes are stained using Lysotracker dye at 2 µM for 1.5 hr at 37 °C in an atmosphere containing 5% CO_2 and then fixed with either 10% formalin or 4% paraformaldehyde (PFA). Confocal images are acquired and finally analyzed using image analysis scripts featuring appropriate algorithms for lysosomes detection and subcellular localization[4].

3. Green Fluorescent Protein Expressing *Mycobacterium tuberculosis* H37Rv (GFP-H37Rv) Culture Conditions

For long term storage, GFP-H37Rv were frozen in D-PBS (around 1 x 10^8 mycobacteria per vial).

1. Resuspend one frozen-stock vial of GFP-H37Rv in an Erlenmeyer flask containing 50 ml of 7H9 broth medium supplemented with Middlebrook OADC enrichment 10%, Glycerol 0.5%, Tween-80 0.05%, and Hygromycin B (50 µg/ml).
2. Incubate 8 days at 37 °C.
3. Measure OD_{600} of the GFP-H37Rv culture.
4. Dilute the GFP-H37Rv culture to obtain OD_{600} = 0.1 in fresh 7H9 broth medium supplemented with Middlebrook OADC enrichment 10%, Glycerol 0.5%, Tween-80 0.05% and Hygromycin B (50 µg/ml).
5. Incubate the GFP-H37Rv at 37 °C for 8 days more before use for the assay.

4. Human Peripheral Blood Monocyte Cells Purification from Whole-blood or Buffy-coat Preparation

1. Dilute the blood pouch 2x in 1x D-PBS (free from $MgCl_2$ and $CaCl_2$) containing 1% FBS.
2. Isolate the monocytes by Ficoll density gradient centrifugation at 400 x g for 20 min.
3. Collect the isolated monocytes.
4. Wash the monocytes 3x with 1x D-PBS (free from $MgCl_2$ and $CaCl_2$) containing 1% FBS by centrifugation at 400 x g for 10 min at room temperature.
5. Concentrate the cells up to 1 x 10^7 cell/ml.
6. Purify monocytes using CD14-magnetic beads according to the manufacturer's protocol (see Materials).
7. After CD14-monocytes purification, seed the cells at 1.5 x 10^6 cell/ml in RPMI 1640 complemented with 10% FBS and 40 ng/ml of human Macrophages Colony Stimulating Factor (hM-CSF) and incubated for 4 days at 37 °C in an atmosphere containing 5% CO_2.
8. After 4 days replace the medium with fresh RPMI 1640 complemented with 10% FBS and 40 ng/ml of hM-CSF and incubated for 2 days at 37 °C in an atmosphere containing 5% CO_2.

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9. After 6 days, the cells can be used for the assay.

Representative Results

High-throughput genome-wide siRNA screening

Mtb is able to colonize immune cells *in vitro* as well as several other lung epithelial cells. For instance, *Mtb*is able to infect and damage A549 epithelial cells that are commonly used as a model for human type II pneumocytes[5-7]. Dectin-1 was reported as a host cell receptor involved in *Mtb* uptake, proinflammatory response and antibacterial effect on intracellular mycobacterial growth in A549 cells[8]. siRNA condition described in Protocol 1 led to 85% of silencing efficiency (data not shown). Silencing Dectin-1 expression with siRNA led to a decrease of intracellular mycobacteria amount in A549 cells. Indeed, after 3 days of silencing and 5 days of infection, the percentage of infected cells is reduced twice in Dectin-1 silenced A549 cells compared to cells transfected with nontargeting scramble siRNA (**Figures 2A** and **2B**). We applied sample-based normalization of siRNA targeted Dectin-1 compared to scramble to define Z-score. As shown in **Figure 2C**, we obtained a Z-score average around -15 for siRNA targeted Dectin-1. Dectin-1 can be used as positive control for the siRNA screen to discover other novel host factor involved in *Mtb* colonization in pneumocytes that could have the same phenotype as that with the Dectin siRNA. The use of a siRNA impacting on the phenotype as a control on each microplate during the screen allows normalization of each plate, which is useful when one wants to perform whole genome screen analysis. The statistical parameter Z' was 0.1 using control based normalization on scramble and siRNA targeted Dectin1, which is an acceptable value for the validation of the siRNA screening data.

High-content compound screening

Compound efficiency on intracellular bacterial growth is evaluated by establishing a dose response curve (DRC) and normalized to the reference positive compound and negative compound solvent controls. Representative DRC of two reference compounds, isoniazid (INH) and rifampicin (RIF), active against *Mtb* growth are shown in **Figure 3**. These curves are obtained in human primary macrophages infected by a GFP-expressing *Mtb* H37Rv strain, with readout after 5 days post-infection. DMSO and INH at a final concentration of respectively 1% and 0.1 µg/ml are commonly used as negative and positive controls, providing basal levels of efficiency (0 and 100%) (**Figure 3A**). Following the image analysis process detailed in **Figure 4B**, multiparametric data are extracted from confocal fluorescence images of infected-human macrophages taken by automated confocal microscope. Active compounds impacting on the intracellular replication of *Mtb* in host cells led to a decrease of mycobacterial load, which corresponds to the area of the GFP signal in cells on pictures (**Figure 3B**). The ratio between intracellular bacterial area and total cell area, calculated using image-based analysis software, is plotted in function of compound concentration, which generates the DRC (**Figure 3B**). These curves allow the determination of both the concentration required to decrease the bacterial load by 50% (IC_{50}) and the minimal concentration required to inhibit 99% of the bacterial replication (MIC_{99}) (**Figure 3C**). Z' based on the control DMSO 1% and the control INH 0.1 µg/ml was 0.49. This Z', really close to 0.5, is acceptable to validate this assay.

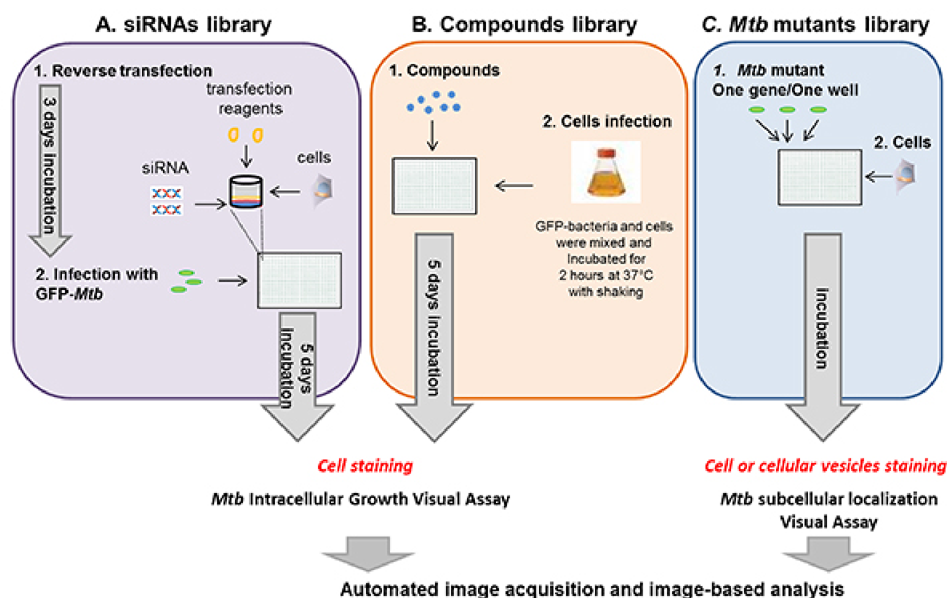

Figure 1. Visual High Content Screening Approaches. Schematic representation of *Mtb* infection model system used for the siRNA (**A**), chemical compounds (**B**) and *Mtb* mutants (**C**) screens. (**A**) siRNA library screen: the cells were transfected with siRNA for 3 days in 384-well plates using reverse transfection method. siRNA transfected cells were infected with GFP-*Mtb* and incubated for 5 days at 37 °C in an atmosphere containing 5% CO_2. The cells were then stained and images were collected using an automated confocal microscope. (**B**) Compounds library screen: the compounds were distributed in 384-well plates. Cell suspension and GFP-*Mtb* were incubated together for 2 hr at 37 °C. Infected cells were seeded in the plates and incubated for 5 days at 37 °C in an atmosphere containing 5% CO_2. The cells were then stained and images were collected using an automated confocal microscope. (**C**) Fluorescent-*Mtb* mutant library: the fluorescent *Mtb* mutants were seeded in 384-well plates and the host cell suspension was distributed. The incubation time varied depending on the assay. The cells or cellular vesicles were stained before automated image acquisition. Click here to view larger image.

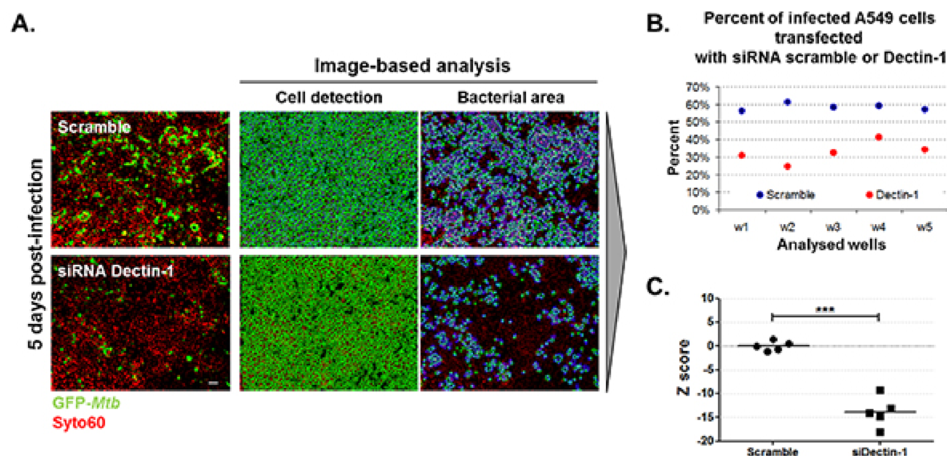

Figure 2. Dectin-1 silencing impacts on Mtb colonization in A549 cells. (**A**) Representative confocal images (10X air lens) of A549 cells transfected with nontargeting siRNA (scramble) or with siRNA specific for Dectin-1 and infected with GFP-*Mtb* H37Rv (MOI5) for 5 days. The scale bar represents 200 µm (A) GFP-*Mtb* H37Rv were visualized in green and the cells in red. The number of cells (**A.** Cell detection) and the intracellular GFP-*Mtb* H37Rv load (**A.** Bacterial area) were determined using image-based analysis software. (**B**) Graphic representation of the percentage of infected A549 cells in 5 replicates (w1 to w5) of Scramble siRNA (blue circles) and Dectin-1 siRNA (red circles). (**C**) Graphic representation of Z-score average of Scramble siRNA and Dectin-1 siRNA. (*** p-value < 0.0001). Click here to view larger image.

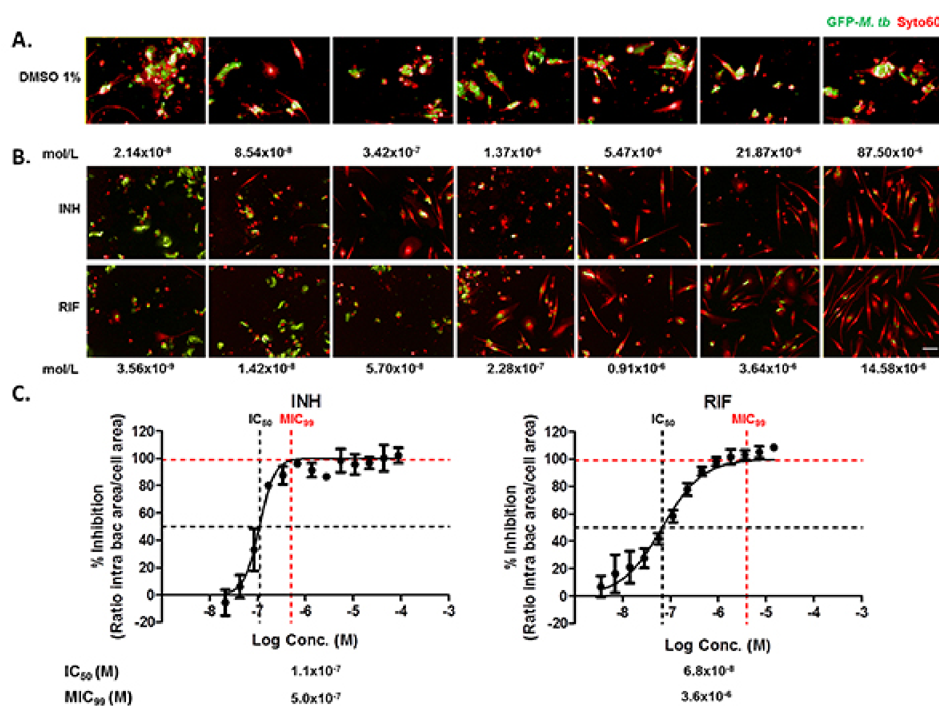

Figure 3. Dose response curve of active reference compounds against Mtb in human macrophages. (**A** and **B**) Representative confocal images (20X water lens) of human macrophages (red, cell labeled with red fluorescent dye) infected with GFP-*Mtb* H37Rv (green) with a MOI1 for 5 days. The scale bar represents 50 µm. (**A**) Images of infected-cells incubated with DMSO 1% used as negative control in the assay. (**B**) Images of infected cells incubated with increasing concentration of two reference compounds isonazid (INH) and rifampicin (RIF). (**C**) Dose-response curves (DRC) of INH and RIF. Image-based analysis allowed determination of the DRC for each compound tested. DRC represents the ratio between intracellular GFP-bacterial area and total cell area (Y axis), in function of the compound concentration (log scale, x-axis). In each graph, the DRC of compound was normalized to that of the negative control DMSO 1% (0% inhibition) and the positive control INH at a concentration of 0.1 µg/ml (100% inhibition). For each compound, the concentration required to inhibit 50% of the bacterial colonization (IC_{50}) and the minimal inhibitory concentration (MIC_{99}) were calculated from the DRC. Click here to view larger image.

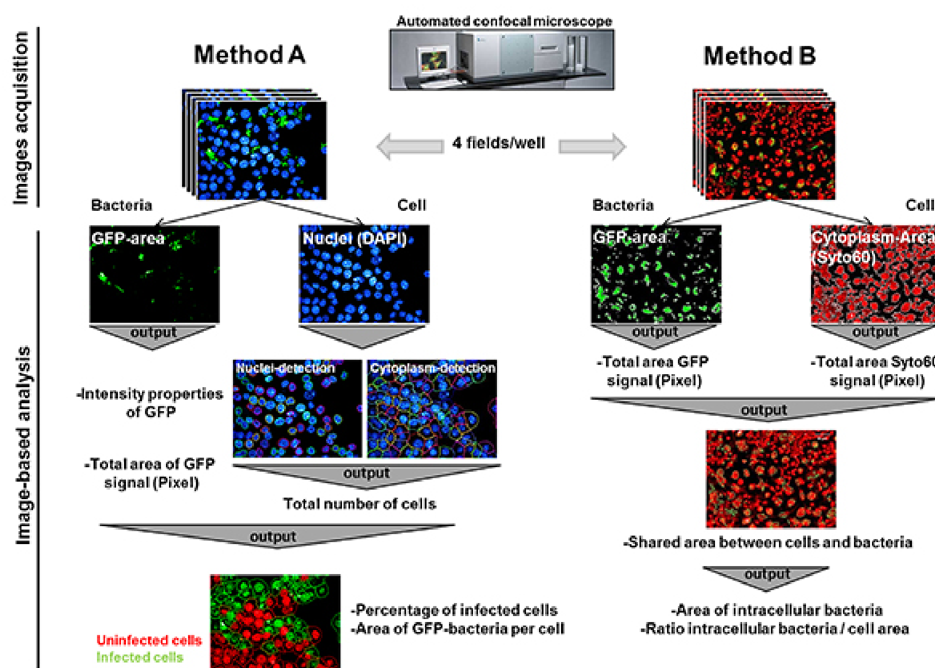

Figure 4. Standard image-based analysis to determine fluorescent intracellular mycobacteria. Images from 384-well plates were acquired using an automated confocal microscope. In this case, 4 different images of the same well (fields) were recorded. Each field was then analyzed using image-based analysis software Acapella 2.6 (Perkin Elmer). (**A**) Each field contained two channels (two colors), one for the bacteria (green) and one for the cell nuclei (blue channel), that were segmented using the following algorithm: *i*) nuclei detection using a built-in Acapella procedure, *ii*) cytoplasm detection, based on the nuclei population, using a built-in Acapella procedure, *iii*) bacteria detection by keeping only pixels which intensity are higher than a manually-defined threshold, *iv*) merging cells position with bacteria position to identify infected cells. Final results, expressed as an average of the four fields, are the total bacterial area, the total number of cell, the percentage of infected cells and the bacterial area per cell (average of all infected cells). (**B**) Each field contained two channels, one for the bacteria (green channel) and one for the cell nuclei and cytoplasm (far-red channel), that were segmented using the following algorithm: *i*) filtering the original channel using an anti-median filter, *ii*) keeping only pixels which intensity are higher than a manually-defined threshold (each channel has its own threshold), *iii*) counting the remaining number of pixel for each channel, *iv*) merging channels and counting the number of pixel shared by both bacteria and nuclei to quantify intracellular bacteria. Final results, expressed as an average of the four fields, are the total bacterial area, the total cellular area, the total area of intracellular bacteria and the ratio between intracellular bacterial area and total area of cells. Click here to view larger image.

Discussion

We describe here the methods required for a phenotypic assay using a GFP-expressing *Mtb* H37Rv strain to infect fluorescently labeled host cells, which makes it appropriate for High-content/High-throughput screens. This protocol could be applied to a broad range of compounds, fluorescent probes and *Mtb* mutants. For each protocol described above, fixation and immunolabeling steps could be performed prior to image acquisition. We use an automated fluorescent confocal microscope equipped with a 20X (NA 0.70) or 60X (NA 1.2) water lens to acquire images. The confocal microscope is equipped with 405, 488, 561, and 640 nm excitation lasers. The emitted fluorescence is captured using 3 cameras associated with a set of filters covering a detection wavelength ranging from 450-690 nm. It is important to note that the adjustment of the microscope excitation and emission settings depends on the type of dyes or fluorochromes used in each experiment. For phenotypic assays, DAPI or Hoechst are commonly used at 5 µg/ml for 10 min to stain nuclei. However, cells can also be stained with different fluorescent dyes specific for the detection of cytoplasm, membrane or cytoskeleton. After image acquisition, pictures should be analyzed using an image-based analysis software. Cells segmentation algorithms based on intensity of each pixel should be used to respectively ascertain the number of cells or the intracellular bacterial area (see **Figure 4**). The generated data should be weighed against a statistically based acceptance criteria to validate the robustness and the accuracy of the assay.

Large-scale high-throughput screens are time- and resource-consuming experiments. Therefore, it is of prime importance to assess beforehand the suitability of the assay. The data collected from phenotypic screens were visualized using spreadsheet software like Excel and generally normalized per plate. The most common quality metrics used for both siRNA and small-molecules screens is the Z. The Z is defined with mean and standard deviation of both positive and negative controls[9]. The Z quantifies whether the assay response is large enough to validate its application for a full-scale screen of samples. The range of the Z is negative infinity to 1, with >0.5 as a very good assay, >0 an acceptable assay and <0 an unacceptable assay. Compared to the small-molecule screens for which the strength of the controls allows the validation of the assay with Z > 0.5, the variability of siRNA screens impacts the Z which tend to be lower (frequently between 0.0-0.5)[10]. Indeed, the success of siRNA screens depends on the optimization of *i*) the efficiency of siRNA delivery into the cells, *ii*) the cytotoxicity induced by the transfection and *iii*) the assay condition for the efficiency of gene silencing. The siRNA can easily be transfected in various cell lines, such as HeLa, following the manufacturer's transfection protocol, but efficient siRNA transfection in some cell lines including macrophages still remains a challenge. Nevertheless, viral vector-mediated expression of short hairpin RNAs (shRNA) could represent a good alternative[11]. To escape the difficulty

of interpretation, the data collected from siRNA screens are frequently normalized relative to the normal standard distribution to define the Z-score (also called Z-value) for each point[10,12]. Then, hit-samples were ranked according to the Z-score which typically belongs to less than -2 or more than +2. Finally, selected hits in the primary screen were retested in a secondary screen including more replicates in order to confirm the phenotype observed in the primary screen.

Our protocol could easily be applied for small scale screens with equipment. To achieve this, assay miniaturization in the micro-titer plates and manipulation of library of molecules are needed to optimize the process of conservation, dispensing and mixing[13]. Furthermore, it is recommended to use an automated robotic platform, including dispensers, in order to accurately and reproducibly control assay conditions in the micro-titer plates. The huge amount of data produced by high throughput screening (HTS) should also be managed by database and an adapted pipeline for image analysis of the confocal picture, data storage and transfer. The assays presented in this report were performed in Biosafety Level 3 facility (BSL3). The strict adherence to safety in BSL3 increases the difficulty of the screens. Indeed, many equipment required for the screens must be available in BSL3 and isolated to protect the worker and the environment from contamination. Therefore, the installation of the adapted pipeline in BSL3 required a lot of space. For this reason, our protocol was developed to have a maximum of steps performed out the BSL3 like siRNA transfection and compound transfer to the plates. The cell infection thru image acquisition steps were performed in BSL3 conditions. The images were then transferred in a dedicated server and analyzed out of the BSL3.

The phenotypic assay described here was based on two methods of image analysis (**Figure 4**). The first method, used for siRNA screening, was designed to give the number of infected cells. This parameter was found to efficiently compare the effect of gene silencing on *Mtb* intracellular replication. When screening compounds however, a more basic read-out based on the total area of cells was sufficient to clearly identify active compounds. As this second method is faster and easier to implement, it was preferred for the analysis of images arising from compounds screening. To go further, more fluorescent dyes and/or labeling probes could be added and image analysis scripts could be optimized to generate multiparametric data such as colocalization, nuclei and cell morphology, cell death, bacterial aggregation, as well as intracellular trafficking of the bacteria. It is important to note that for intracellular trafficking or colocalization assays, it is essential to get Z-stacks in order to apply a cumulative assessment.

Disclosures

No conflicts of interest declared.

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