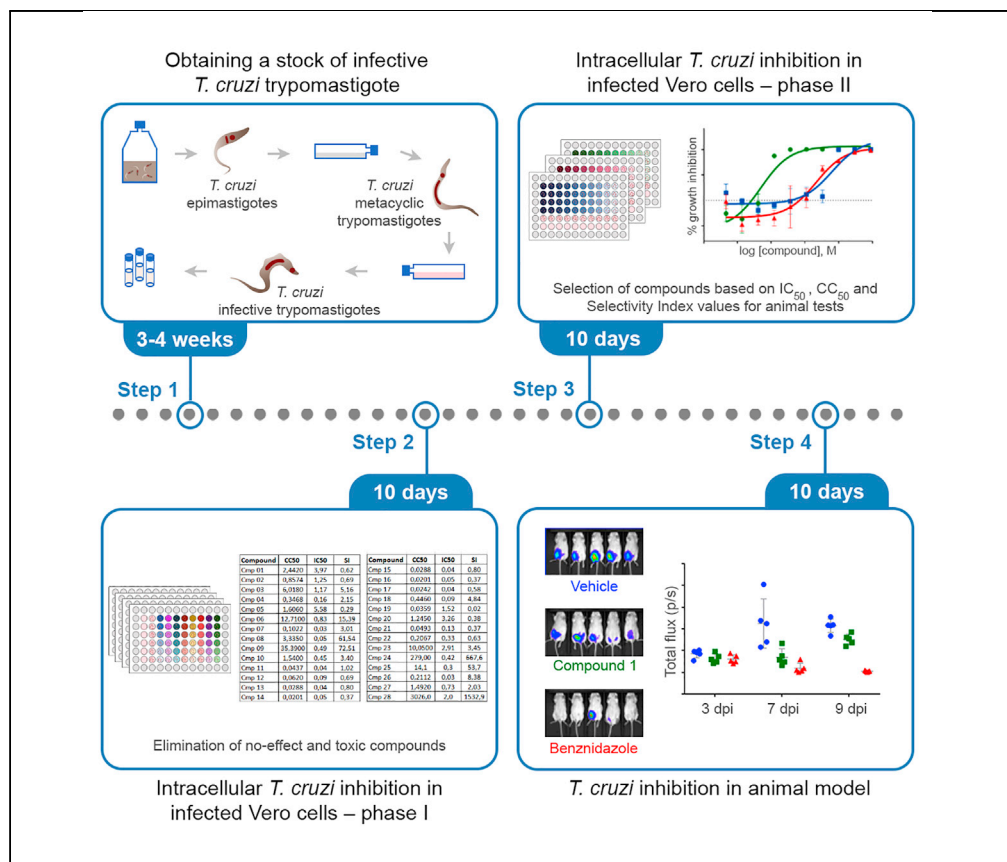


Protocol

In cellulo and in vivo assays for compound testing against *Trypanosoma cruzi*



Eloise Pavão Guerra-Slampo, Gisele Fernanda Assine Picchi-Constante, Martin Marek, Christophe Romier, Wolfgang Sippl, Nilson Ivo Tonin Zanchin

eloise.slampo@fiocruz.br (E.P.G.-S.)
gisele.picchi@fiocruz.br (G.F.A.P.-C.)
nilson.zanchin@fiocruz.br (N.I.T.Z.)

Highlights

In cellulo infection assays for effective compound testing against *T. cruzi*

High-content image analysis to determine selectivity and toxicity indexes

Bioluminescence in vivo infection assays to evaluate compound efficiency

Here, we describe a combined *in cellulo* and *in vivo* approach to identify compounds with higher potential for efficient inhibition of *Trypanosoma cruzi*. Phase I of *in cellulo* assays is designed to exclude inactive or toxic compounds, while phase II is designed for accurate IC₅₀, CC₅₀, and selectivity index (SI) determination. Compounds showing high SI are tested using *in vivo* infection models in parallel with benznidazole to assess their efficacy relative to a reference drug used for Chagas disease treatment.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

In cellulo and *in vivo* assays for compound testing against *Trypanosoma cruzi*

Eloise Pavão Guerra-Slompo,^{1,6,7,*} Gisele Fernanda Assine Picchi-Constante,^{1,6,7,*} Martin Marek,^{2,3,5} Christophe Romier,^{2,3} Wolfgang Sippl,⁴ and Nilson Ivo Tonin Zanchin^{1,8,*}

¹Instituto Carlos Chagas, Fiocruz Paraná, Curitiba, Paraná 81350-010, Brazil

²Université de Strasbourg, CNRS, INSERM, Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR 7104, U 1258, 67404 Illkirch, France

³IGBMC, Department of Integrated Structural Biology, 1 rue Laurent Fries, B.P. 10142, 67404 Illkirch Cedex, France

⁴Institute of Pharmacy, Martin-Luther-Universität Halle-Wittenberg, Wolfgang-Langenbeck-Straße 4, 06120 Halle/Saale, Germany

⁵Present address: Loschmidt Laboratories, Department of Experimental Biology & RECETOX, Faculty of Science, Masaryk University, Kamenice 5/C13, 625 00 Brno, Czech Republic

⁶These authors contributed equally

⁷Technical contact

⁸Lead contact

*Correspondence: eloise.slompo@fiocruz.br (E.P.G.-S.), gisele.picchi@fiocruz.br (G.F.A.P.-C.), nilson.zanchin@fiocruz.br (N.I. T.Z.)

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SUMMARY

Here, we describe a combined *in cellulo* and *in vivo* approach to identify compounds with higher potential for efficient inhibition of *Trypanosoma cruzi*. Phase I of *in cellulo* assays is designed to exclude inactive or toxic compounds, while phase II is designed for accurate IC₅₀, CC₅₀, and selective index (SI) determination. Compounds showing high SI are tested using *in vivo* infection models in parallel with benznidazole to assess their efficacy relative to a reference drug used for Chagas disease treatment.

For complete details on the use and execution of this protocol, please refer to Marek et al. (2021).¹

BEFORE YOU BEGIN

Compound testing against *T. cruzi* using cell infection assays is very laborious and time consuming due to the delicate nature of cell cultures, short lifetime of *T. cruzi* infective stage and to the requirement of many replicates for acquisition of reproductive and conclusive data. Although some steps can be performed using automated liquid handling systems, automation is not available in most laboratories. Therefore, this protocol describes the procedures for meticulous handling of cell cultures, *T. cruzi* infective forms and compound preparation for testing. It involves many intense and precise manual pipetting steps. This, together with culture plate organization and cell imaging analyses are described and illustrated in detail to facilitate reproducibility. Considering that many compounds may have inhibitory effect in tissue culture but are ineffective *in vivo*, testing compounds in animal models is a crucial step for the development of new drugs. However, due to both ethical and practical reasons, compound testing in animal infection models is only feasible for a very limited number of compounds. In this context, the rationale used in this protocol is intended to provide the basis to quickly exclude the compounds that are inactive against *T. cruzi* or toxic to the host cells. For the compounds that pass this phase, the protocol describes a deeper investigation, still using *in cellulo* assays to select the set of compounds with higher selective index that are worth testing in animal infection models. The test compounds are evaluated in mice infection assays in parallel



with a reference drug. Based on the performance of the test compounds relative to the reference drug a decision can be made on which compounds should be tested in clinical trials.

△ **CRITICAL:** All cell manipulations should be carried out under strict aseptic conditions and all solutions should be sterile.

Institutional permissions

National and Institutional biosafety requirements for *T. cruzi* studies can vary substantially worldwide and should be followed considering specific local rules. Herein, parasite manipulations required biosafety level 2 facilities.

Besides that, *in vivo* experiments require previous approval of the project by an animal experimentation ethics committee. Herein, all animal procedures were approved by the Animal Care and Testing Committee of the Tuiuti University of Paraná (CEUA-UTP No. 006-18) and follow the current ethical regulations.

Maintaining *T. cruzi* cultures

⌚ Timing: 3–4 days

T. cruzi Dm28c strain expressing firefly luciferase² is cultured in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) without agitation at 28°C.

1. Cells are passed every 3–4 days or when the cell density reaches around 2×10^7 cells/mL.
 - a. Using a hemocytometer count *T. cruzi* cells and transfer the appropriate amount to have 1×10^6 cells/mL density to a new sterile flask containing LIT medium supplemented with 10% FBS.

Maintaining Vero cells

⌚ Timing: 3–4 days

Vero cells are cultured in complete culture medium at 37°C in a humid 5% CO₂ environment.

2. Cells are passed every 3–4 days following ATCC recommendations in “Detailed product information / Handling information / Subculturing procedure” section at ATCC website.

Alternatives: Other host cells, like the Myoblastic Cell Line, can be used. However, caution should be taken with certain cell lineages (e.g., L929) which may not be as flat as Vero cells when adhered. As a consequence, intracellular parasites may be distributed throughout multiple focal planes, therefore requiring more images from each field to be taken and analyzed.

Obtaining Vero cells for infection experiments

⌚ Timing: 20 min

3. Use Vero cells cultures at 80%–90% confluency (corresponds to a 3-days culture from a seeding density of 6.5×10^4 cells/cm²).
4. Discard the culture medium. Rinse the cells, for each 75 cm² flask use 10 mL of DPBS.
5. Add 2 mL of 0.05% trypsin-EDTA solution to detach the cells and incubate at 37°C for 5 min in a humid 5% CO₂ environment.
6. Under an inverted microscope, observe the cells: if they are dispersed add 6 mL of complete culture medium.
 - a. If they are still attached, incubate 2 min more.

7. Aspirate the cells by gently pipetting and transfer to a 15 mL conical tube.
8. Centrifugate at $300 \times g$ for 5 min.
9. Discard the supernatant and suspend cells in 2 mL of complete culture medium.
10. Count the cells and use the appropriate volume for the experiments immediately.

△ **CRITICAL:** For infection assays, it is important not to use cultures with more than 12 passages. (See [troubleshooting - problem 1](#)).

Preparing compound solutions for cell infection assays

⌚ **Timing:** 2 h

11. Dilute all compounds in DMSO at a final concentration of 100 mM.

△ **CRITICAL:** It is important to verify drugs solubility information in manufacturer's recommendation about drug solubility in its data sheet.

Alternatives: If any compound does not solubilize at a concentration of 100 mM in DMSO, it is possible to decrease the concentration to about 20 mM and/or to change the solvent to polyethylene glycol 200, ethylene glycol, 2-propanol or methanol.

12. Distribute the appropriate volume according to the number of replicates.

Note: In this protocol, 10 μ L aliquots in 0.2 mL tubes were used.

13. Store at -80°C until use.

Obtaining mice for infection and compound testing

⌚ **Timing:** 8–12 weeks

Groups of five 8–12-weeks old mice are recommended for each compound to be tested. It is also recommended to have five mice for an untreated control group and five mice to be treated with a reference drug. This age choice considers adult animals as being in the appropriate immunological developmental range for *T. cruzi* infection and compound response assays. Older animals may present different drug response due to aging-related issues like reduced absorption, slower drug metabolization and other hormonal interferences.

In this protocol, Balb/C females were used due to their small size and lower aggressiveness for easier handling. As the literature shows, there are some differences in infection/treatment responses or luminescence intensity detection regarding the sex of animals.³ Considering that this protocol uses a minimal number of animals, the possibility of the occurrence of individual variability should be minimized to keep assay heterogeneity as low as possible.

Other mice strains, like Swiss, are also used as infection models and could be used in this assay. However, the use of pigmented fur or skin animals should be avoided in bioluminescence assays to prevent signal blockage. Additional literature should be checked for potential interference in bioluminescence imaging of mice.^{4,5}

△ **CRITICAL:** Obtain approval for the use of animals from an official Animal Experimentation Ethics Committees before starting animal experiments.

Preparing compounds and reference drug for mice treatment

⌚ Timing: 2 h

14. If starting from lyophilized compounds, weight the powder and prepare as many separate aliquots as the number of required doses for all treatments.
 - a. For benznidazole: (100 mg per kg per day) × (number of animals + 2*). Prepare a separate tube for each day of treatment.
 - b. The doses of the test compounds should be determined according to their respective *in cellulo* IC₅₀ and decay kinetics in animal model. Compounds: (defined dose per kg) × (number of animals + 2*). Prepare as many separate tubes as the number of doses.

Note: For rapidly processed compounds, injections every 12 h should be considered.

Note: * extra volume needed to compensate for dead syringe volume and volume adjustment in the syringe.

15. Store the weighted dry aliquots at –20°C and dissolve them just moments prior to the administration as a precaution to preserve activity.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
2-Hydroxypropyl-β-cyclodextrin	Sigma	Cat# 332607
Benznidazole (N-benzyl-2-nitro-1H-imidazole-1-acetamide)	Sigma	Cat# 419656
D-luciferin, potassium salt	Biovision	Cat# 7903-100
DAPI	Sigma	Cat# D9542
DMEM low glucose medium	Gibco	Cat# 31600034
DMSO	Sigma	Cat# D2650
DPBS	Gibco	Cat# 21600010
Evans blue	Sigma	Cat# E2129
Fetal bovine serum (FBS)	Gibco	Cat# 12657029
GlutaMAX Supplement	Gibco	Cat# 35050061
Liver infusion tryptose (LIT) medium	Prepared in house	Camargo ⁶
Paraformaldehyde	Sigma	Cat# P6148
PBS pH 7.4	Gibco	Cat# 70011044
Penicillin-Streptomycin	Gibco	Cat# 15140148
TAU3AAG medium	Prepared in house	Bonaldo et al. ⁷
TB56	Synthesized	Marek et al. ¹
Triatomine artificial urine (TAU) medium	Prepared in house	Bonaldo et al. ⁷
Trypsin-EDTA (0.5%) solution	Gibco	Cat# 15400054
Experimental models: Cell lines		
Vero cells	ATCC	Cat#CCL-81
Experimental models: Organisms/strains		
Balb/C mice, female, 8–12-weeks old	Provided and maintained by Laboratory of Animal Models - LACEA, from Fiocruz Paraná	N/A
<i>Trypanosoma cruzi</i> Dm28c strain	Stock from Fiocruz Paraná	Contreras et al. ⁸
<i>Trypanosoma cruzi</i> Dm28c strain expressing firefly luciferase	Provided by Wanderley de Souza's lab, from UFRJ	Bonaldo et al. ²

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism Ver. 7.05	GraphPad	GraphPad Prism
Harmony Software Ver. 4.8	PerkinElmer	Harmony
Living Image Software Ver. 4.3	PerkinElmer	Livingx Image
Other		
96-well culture plates	Jet Bio-Fil	Cat# TCP011096
CoolCell container	Corning	Cat# 432000

MATERIALS AND EQUIPMENT

Cell dye solution

PBS solution containing 1 $\mu\text{g}/\text{mL}$ of DAPI and 0.001% (v/v) of Evans Blue dye.

Calculate the total volume using: $100 \mu\text{L} \times (\text{number of wells to be used per plate}) \times (\text{number of plates}) \times 1.2$ (20% extra volume). DAPI stains nucleic acids and the Evans Blue dye stains cells cytoplasm.

Prepare freshly at the time of use.

Complete culture medium

Reagent	Final concentration
DMEM low glucose medium	90%
FBS	10%
Penicillin	to - 10 units/mL
Streptomycin	100 $\mu\text{g}/\text{mL}$

Store at 4°C. Stable for a month. To use after a month, add GlutaMAX Supplement or supplement periodically with 2 mM L-glutamine.

Alternatives: Other culture medium as RPMI can be used, however it should be changed more frequently specially during release of trypomastigotes in order to avoid their differentiation into amastigotes.

△ CRITICAL: For Dm28c strain of *T. cruzi*, it is essential that the culture medium has a low glucose concentration (1 g/L) to obtain high levels of infection in cell culture assays.

Inoculation vehicle

Reagent	Final concentration
DMSO	7%
2-Hydroxypropyl- β -cyclodextrin	40%
PBS	to 100 $\mu\text{L}/\text{dose}$

Store at -80°C . Stable for several days.

Liver infusion tryptose - B (LIT B) medium

Reagent	Final concentration
NaCl	68 mM
KCl	5 mM
Na_2HPO_4	56.4 mM
Glucose	10 mM
Bacto-tryptose	0.5%
Liver Infusion	0.5%

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Reagent	Final concentration
Yeast extract	1.5%
Hemin 2%	0.0025%
inactivated Fetal Bovine Serum	10%

Adjust to pH 7.2 with HCl. Filter sterilely. Store at 4°C. Stable for several months.

TAU3AAG medium

Reagent	Final concentration
NaCl	190 mM
KCl	17 mM
MgCl ₂	2 mM
CaCl ₂	2 mM
L-proline	8 mM
Phosphate buffer pH 6.8	8 mM
Sodium bicarbonate	0.035% (w/v)
Sodium L-glutamate	50 mM
Sodium L-aspartate	2 mM
Glucose	10 mM
ddH ₂ O	to 100 mL

Adjust to pH 6 with HCl. Filter sterilely. Store at 4°C. Stable for several months.

Triatomine artificial urine (TAU) medium pH 6.0

Reagent	Final concentration
NaCl	190 mM
KCl	17 mM
MgCl ₂	2 mM
CaCl ₂	2 mM
Phosphate buffer pH 6	8 mM
Sodium bicarbonate	0.035% (w/v)
ddH ₂ O	to 10 mL

Adjust to pH 6 with HCl. Filter sterilely. Store at 4°C. Stable for several months.

STEP-BY-STEP METHOD DETAILS

⚠ **CRITICAL:** All cell manipulations should be carried out under strict aseptic conditions and all solutions should be sterile.

Preparing a stock of infective *T. cruzi* trypomastigotes

⌚ **Timing:** 3–4 weeks

In this step, cell-derived trypomastigotes will be generated to be used in cell and animal infection experiments (Figure 1).

1. Obtain metacyclic trypomastigotes from *in vitro* differentiation.^{7,9}
 - a. Cultivate epimastigotes of the *T. cruzi* Dm28c strain expressing firefly luciferase in LIT medium for at least five days until late exponential growth phase (~ 5 × 10⁷ cells/mL);
 - b. Collect 5 × 10⁸ cells by centrifugation at 6,000 × g for 15 min at 20°C–25°C;
 - c. Remove completely the supernatant and resuspend cells in 1 mL of triatomine artificial urine (TAU) medium (density of 5 × 10⁸ cells/mL).

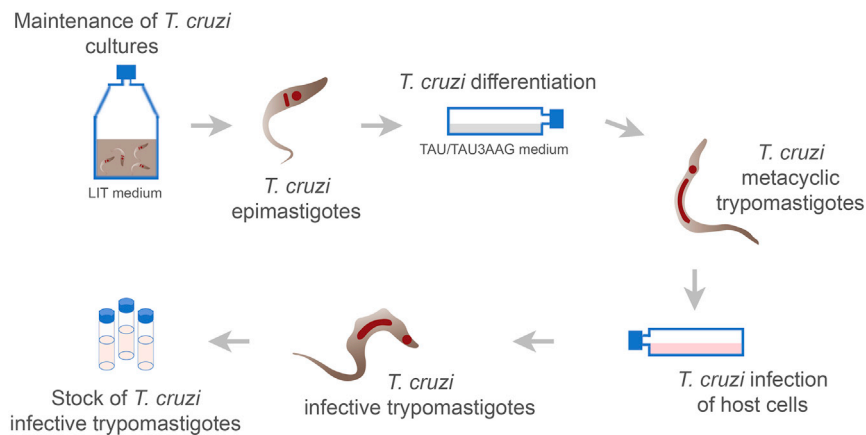


Figure 1. Overview of the preparation of the stock of infective *T. cruzi* trypomastigotes

- i. incubate for 2 h at 28°C to obtain stressed epimastigotes;
- d. Prepare two 75 cm² culture flasks containing 50 mL of TAU3AAG medium each and transfer 500 µL each of the stressed epimastigote suspension (final density of 5×10^6 cells/mL).
 - i. incubate at 28°C for 72 h;
- e. After this time, recover the metacyclic trypomastigotes by centrifugation at $6,000 \times g$ for 20 min at 10°C.
- f. Suspend the metacyclic trypomastigotes in 1 mL of complete culture medium.
- g. Determine the metacyclic trypomastigotes number using a hemocytometer and a light microscope. Proceed immediately to the next step.
2. Infect Vero cells with metacyclic trypomastigotes using a MOI of 100.
 - a. Mix 5×10^5 Vero cells (see [preparing Vero cells for infection experiments](#)) with 5×10^7 metacyclic trypomastigotes recovered in the previous step.
 - b. Divide the mix of cells and parasites in two 75 cm² culture flasks, complete the volume to 10 mL with complete culture medium and incubate at 37°C in a humid 5% CO₂ environment.
 - c. After 24 h of infection, discard the medium and rinse 2 times with 10 mL of DPBS to remove all trypomastigotes that have not invaded the cells.
 - d. Add 10 mL of complete culture medium to each flask and incubate at 37°C in a humid 5% CO₂ environment.
 - e. After 72 h of infection, replace the medium with fresh 10 mL of complete culture medium and incubate at 37°C in a humid 5% CO₂ environment.
 - f. After 96 h of infection, recover cell-derived trypomastigotes by centrifugation at $3,000 \times g$ for 20 min at 12°C.
 - i. Suspend parasites in 2 mL of complete culture medium and determine the trypomastigotes number as described above.

△ CRITICAL: The first release of cell-derived trypomastigotes after an infection of Vero cells with metacyclic trypomastigotes can take more than 96 h. If the number of released parasites is less than 5×10^6 cells, add fresh 10 mL of medium to culture flasks and continue to incubate, monitoring daily until enough trypomastigotes have been released into the culture medium. See also [troubleshooting section \(problem 2\)](#).
3. Prepare infective cell-derived trypomastigotes using a MOI of 10.
 - a. Mix 5×10^5 Vero cells (see [preparing Vero cells for infection experiments](#)) with 5×10^6 cell-derived trypomastigotes in a total of 20 mL of complete culture medium.
 - b. Divide into two 75 cm² culture flask and incubate for 4 h at 37°C in a humid 5% CO₂ environment.

- c. Discard the medium and rinse 2 times with 10 mL of DPBS to remove all free trypomastigotes.
- d. Add 10 mL of complete culture medium and incubate at 37°C in a humid 5% CO₂ environment.
- e. After 72 h of infection, replace the medium with a fresh 10 mL of complete culture medium and incubate again at 37°C in a humid 5% CO₂ environment.
- f. After 96 h of infection, recover the cell-derived trypomastigotes by centrifugation at 3,000 × g for 20 min at 12°C.

△ **CRITICAL:** To achieve higher infection rates for the intended assays, perform at least three cycles of reinfection with cell-derived trypomastigotes before storage. See also [troubleshooting section \(problem 2\)](#).

4. Store infective cell-derived trypomastigotes in liquid nitrogen.
 - a. Suspend the cell-derived trypomastigotes in the appropriate volume of 10% DMSO in FBS (v/v), to reach a concentration of 5 × 10⁶ cells/mL.
 - b. Transfer 1 mL to each cryotube.
 - c. Put the cryotubes in a CoolCell container and place it in a –80°C freezer for 24 h.

Alternatives: If a CoolCell container or similar is not available, it is possible to freeze the cryotubes in a small Styrofoam box for 4 h at –20°C and then for 20 h at –80°C.

- d. Store the frozen cryotubes under liquid nitrogen.

△ **CRITICAL:** Repeat steps 3 and 4 until there are at least 30 frozen vials of 5 × 10⁶ cell-derived trypomastigotes. This is particularly important to keep experiments running continuously without having to prepare cell-derived trypomastigotes for each round of testing. Alternatively, it is possible to expand the number of flasks at the same time to have more cell-derived trypomastigotes, minimizing batch to batch variations.

Compound testing in cell infection assays – Phase I: Preliminary screening for exclusion of non-active and toxic compounds

⌚ **Timing:** 10 days

In this step, the compounds are tested using cell infection assays in a single curve with a sparse number of concentration points. Compound testing in cell infection assays is very laborious and time consuming. Therefore, this approach is particularly interesting for a first screen, when a large number of compounds must be tested, allowing for exclusion of compounds non-active against *T. cruzi* and of compounds toxic for the host cells in parallel with pre-selection of those with higher intracellular *T. cruzi* inhibition for deeper investigation of their activity profile ([Figure 2](#)).

△ **CRITICAL:** Vero cells will be used throughout all the procedure, so it will be necessary always to keep them in culture (see [maintaining Vero cells culture](#)).

Note: In this work, the amount of Vero cells and trypomastigotes are calculated for testing 28 different compounds (a total of 4 plates) but adjustments can be made according to the number of compounds to be tested in parallel.

Alternatives: In this work, all pipetting was done manually, but if available, it is possible to use automatic liquid dispensing robots.

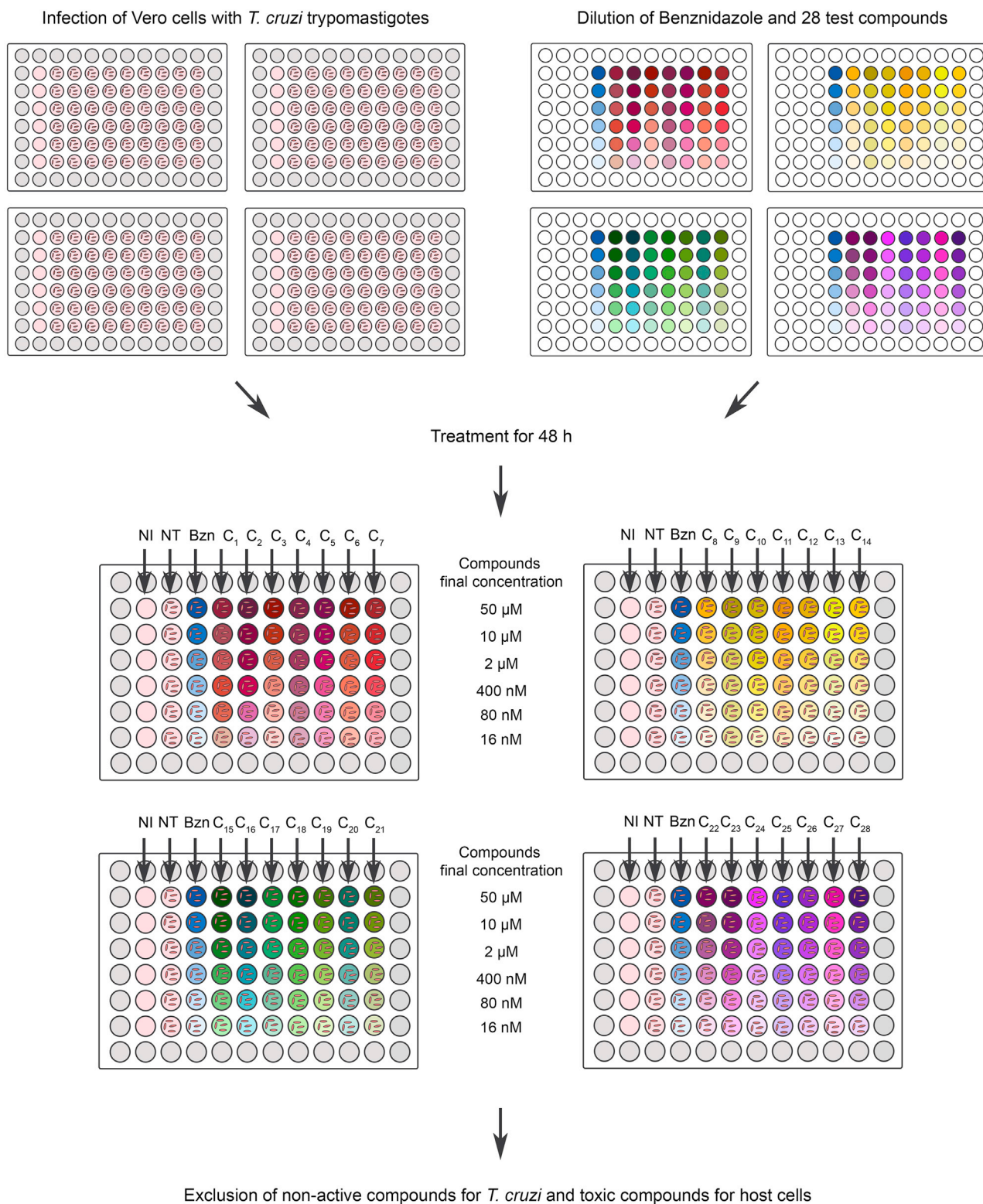


Figure 2. Overview of the compound testing – Phase I strategy

Day 1:

5. Thaw a frozen vial containing 5×10^6 cell-derived trypomastigotes for obtention of fresh infective trypomastigotes.
 - a. Thaw rapidly by gentle agitation in a 37°C water bath and remove just before the content is fully thawed.

Note: To reduce the possibility of contamination, keep the O-ring and cap above the water.

- b. Transfer the trypomastigotes to a centrifuge tube containing 9 mL of complete culture medium and centrifuge at $3,000 \times g$ for 20 min at 12°C.
 - c. Discard the supernatant.
 - d. Suspend the trypomastigotes in 1 mL of complete culture medium.
6. Mix 5×10^5 Vero cells (see [Obtaining Vero cells for infection experiments](#)) with the trypomastigotes (5×10^6 parasites) in a 75 cm² culture flask, complete the volume to 10 mL with complete culture medium and incubate for 4 h at 37°C in a humid 5% CO₂ environment.
7. After 4 h of infection, discard the medium and rinse 2 times with 10 mL of DPBS to remove all the parasites that have not invaded the cells.
8. Add 10 mL of complete culture medium and incubate for 96 h at 37°C in a humid 5% CO₂ environment.

Day 3:

9. In parallel, maintain a Vero cell culture for the next steps.
 - a. Inoculate 3.5×10^5 Vero cells in 10 mL of complete culture medium in a 75 cm² culture flask (see [maintaining Vero cells](#) and [preparing Vero cells for infection experiments](#)).
 - b. Incubate at 37°C in a humid 5% CO₂ environment.

Day 5:

10. At late afternoon, discard the medium of the infected cells (from step 8) and rinse 2 times with 10 mL of DPBS to remove all trypomastigotes that have already been released, aiming to minimize new cycles of infection.
11. Add 10 mL of complete culture medium and incubate at 37°C in a humid 5% CO₂ environment.

Day 6:

12. In the morning, collect the released trypomastigotes by transferring the supernatant to a centrifuge tube.
 - a. Recover the cell-derived trypomastigotes from the supernatant by centrifugation at $3,000 \times g$ for 20 min at 12°C.
 - i. Suspend the parasites in 2 mL of complete culture medium and store at 4°C until the time of use.
 - b. add 10 mL of complete culture medium to the cell layer in the culture flask and incubate at 37°C in a humid 5% CO₂ environment for another cycle of trypomastigote release.
13. Still in the morning, using the cells seeded at step 9 (see [preparing Vero cells for infection experiments](#)), dilute the appropriate quantity of Vero cells in complete culture medium to have 2×10^4 cells/mL ([Figure 3](#)).
 - a. QUANTITY OF CELLS: $2,000$ cells per well \times 60 wells \times 4 plates \times 1.2 (20% extra volume) = 5.76×10^5 cells.
 - b. TOTAL VOLUME: 100 μ L per well \times 60 wells \times 4 plates \times 1.2 (20% extra volume) = 28.8 mL.

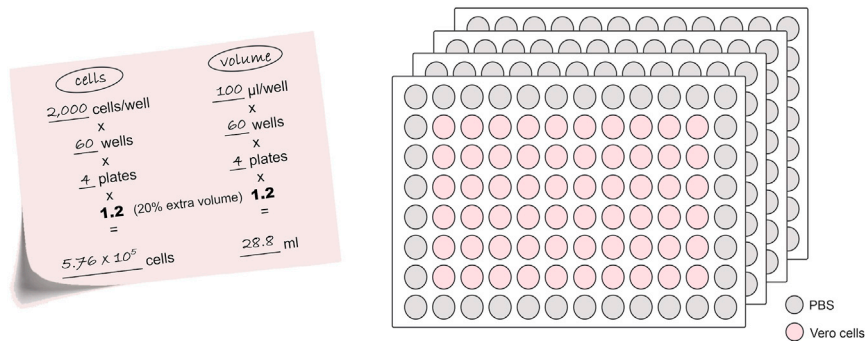


Figure 3. Plating of Vero cells for the compound screening assay

Note: To ensure that the volume will be sufficient until the end of pipetting, it is recommended to make the calculations with an extra volume. In this work, we use a factor of 1.2 (20% of extra volume).

14. Using a multichannel pipette, transfer 100 μL of Vero cells (2×10^4 cells/mL) to each well of four 96-wells culture plates as indicated in [Figure 3](#). Incubate at 37°C in a humid 5% CO_2 environment.
 - a. Do not use the wells of the border rows of the plate as they could not be properly analyzed by the automated imaging system but do not leave them empty. Add 100 μL of PBS at cell-free wells.

Note: If other types of culture plates and automated imaging system provide good images from the edge wells, allowing for proper analysis, it is possible to use the edge wells. However, additional attention should be taken with evaporation issues.

Note: In the steps using a multichannel pipette, use a sterile multichannel pipette tray or a sterile 9 cm diameter culture plate to keep the cell suspension.

15. In the afternoon, recover the cell-derived trypomastigotes from the supernatant of cell culture from step 12b by centrifugation at $3,000 \times g$ for 20 min at 12°C. Suspend parasites in 2 mL of complete culture medium.
16. Count the trypomastigotes from both parasite suspensions (“morning” from step 12a and “afternoon” from step 15) using a hemocytometer and a light microscope.
 - a. Verify if the quantity of trypomastigotes recovered from step 15 (“afternoon” parasites) is sufficient for the total needed in the next step. If not, combine with the trypomastigotes recovered from step 12a (“morning”).

△ CRITICAL: If the total quantity of parasites is not enough for the next step, abort the experiment and restart from step 5 (see also [problem 2](#)).

17. Infect Vero cells with a MOI of 20. For this, dilute the appropriate quantity of trypomastigotes in complete culture medium to a concentration of 4×10^5 trypomastigotes/mL ([Figure 4](#)).
 - a. QUANTITY OF TRYPOMASTIGOTES: 20 (MOI) \times $2,000$ cells per well \times 54 wells \times 4 plates \times 1.2 extra volume = 1.0368×10^7 trypomastigotes.
 - b. TOTAL VOLUME: 100 μL per well \times 54 wells \times 4 plates \times 1.2 extra volume = 25.92 mL.
18. Using a multichannel pipette, transfer 100 μL of trypomastigotes (4×10^5 trypomastigotes/mL) to each cell-containing well of the 96-well plate, except for the wells of the plate border rows and for the wells of non-infected control cells as indicated in [Figure 4](#).
19. Add 100 μL of complete culture medium to the non-infected control wells.

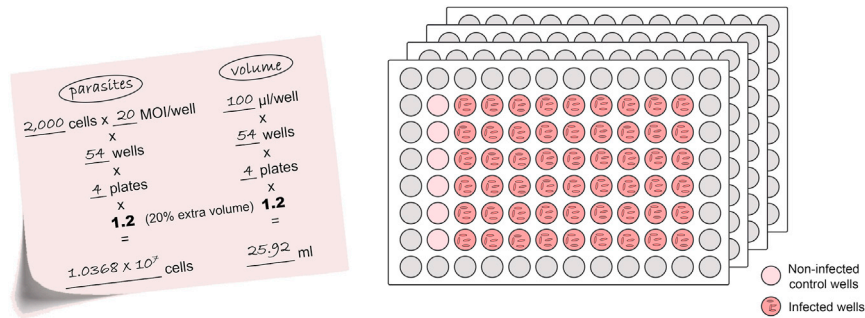


Figure 4. Infection of cells for screening assay

20. Incubate the plates for 16 h–20 h at 37°C in a humid 5% CO₂ environment.

Day 7:

21. After 16 h of infection, carefully discard the supernatant by aspirating with a pipette. Rinse each well with 150 µL of DPBS to remove all trypomastigotes that have not invaded the cells.

△ CRITICAL: Aspirate and dispense the liquid by touching the tip to the wall of the well and taking care not to touch the bottom as indicated in Figure 5. Pipette slowly so as not to disturb the cell layer.

22. Carefully remove all remaining DPBS. Add 100 µL of complete culture medium to each cell-containing well (Figure 6) and incubate at 37°C in a humid 5% CO₂ environment.

23. Prepare the compounds for treatment:

- a. Using 0.2 mL tubes, dilute the compounds in DMSO so to have a 5-fold dilution curve ranging from 10 mM to 3.2 µM as indicated in Figure 7.
- b. Distribute 100 µL of complete culture medium to four new 96-well.
 - i. add 1 µL of each one of the 28 pre-diluted compounds as indicated in Figure 8. Mix well.

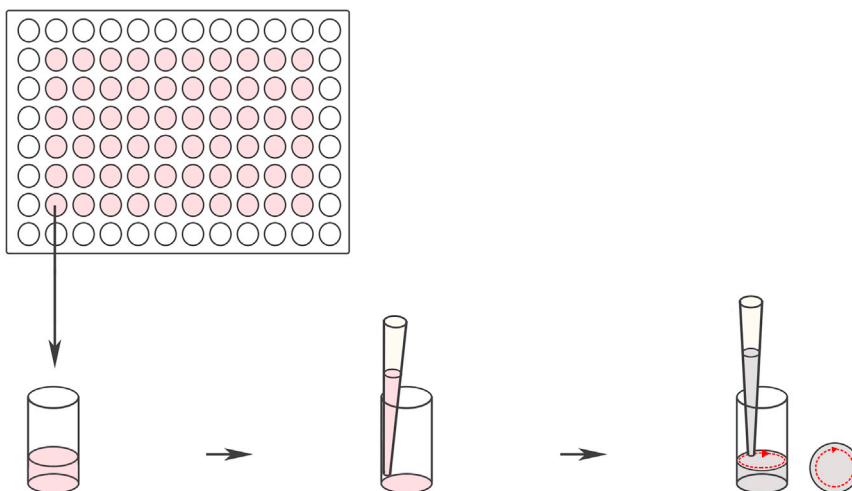


Figure 5. Detail of the washing procedure

Aspirate and dispense the liquid by touching the tip to the wall of the well, in a circular movement to ensure complete washing, and taking care not to touch the bottom. Pipette slowly so as not to disturb the cell layer.

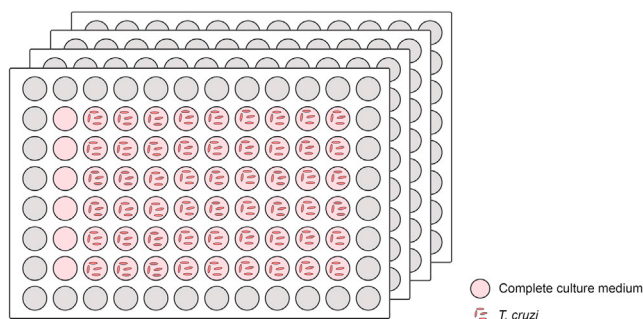


Figure 6. Addition of complete culture medium after washing

△ **CRITICAL:** It is not recommended to use more than 1% final DMSO in the cultures since it can be toxic to cells.

Alternatives: To reduce pipetting errors of small volumes, it is possible to increase the volumes as long as the final DMSO concentration in the cultures is maintained at a maximum of 1%.

24. Treat cells (from step 22) as indicated in [Figure 9](#) using a different set of compounds (BZN + 7 test compounds) for each culture plate from step 22.
 - a. to the non-infected and untreated control wells, add 100 μL of 1% DMSO in complete culture medium.
 - b. to the reference drug (BZN) and compound test wells, add 100 μL of the dilutions prepared in the step 23b.

Note: At this point each well contains 200 μL of culture medium and 0.5% of DMSO. The compound distribution in the plates with the final dilutions is shown in [Figure 10](#).

25. Incubate all four 96-well culture plates treated with the compounds for 48 h at 37°C in a humid 5% CO_2 environment.

Day 9:

26. After 48 h of treatment, carefully discard the supernatant by aspirating with a pipette.
 - a. Rinse each well with 150 μL of DPBS as indicated in [Figure 5](#).
27. Add 100 μL of 4% paraformaldehyde in DPBS and incubate for 15 min at 20°C–25°C.

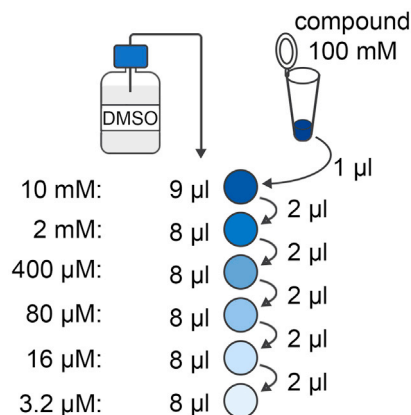


Figure 7. Pre-dilution of compounds

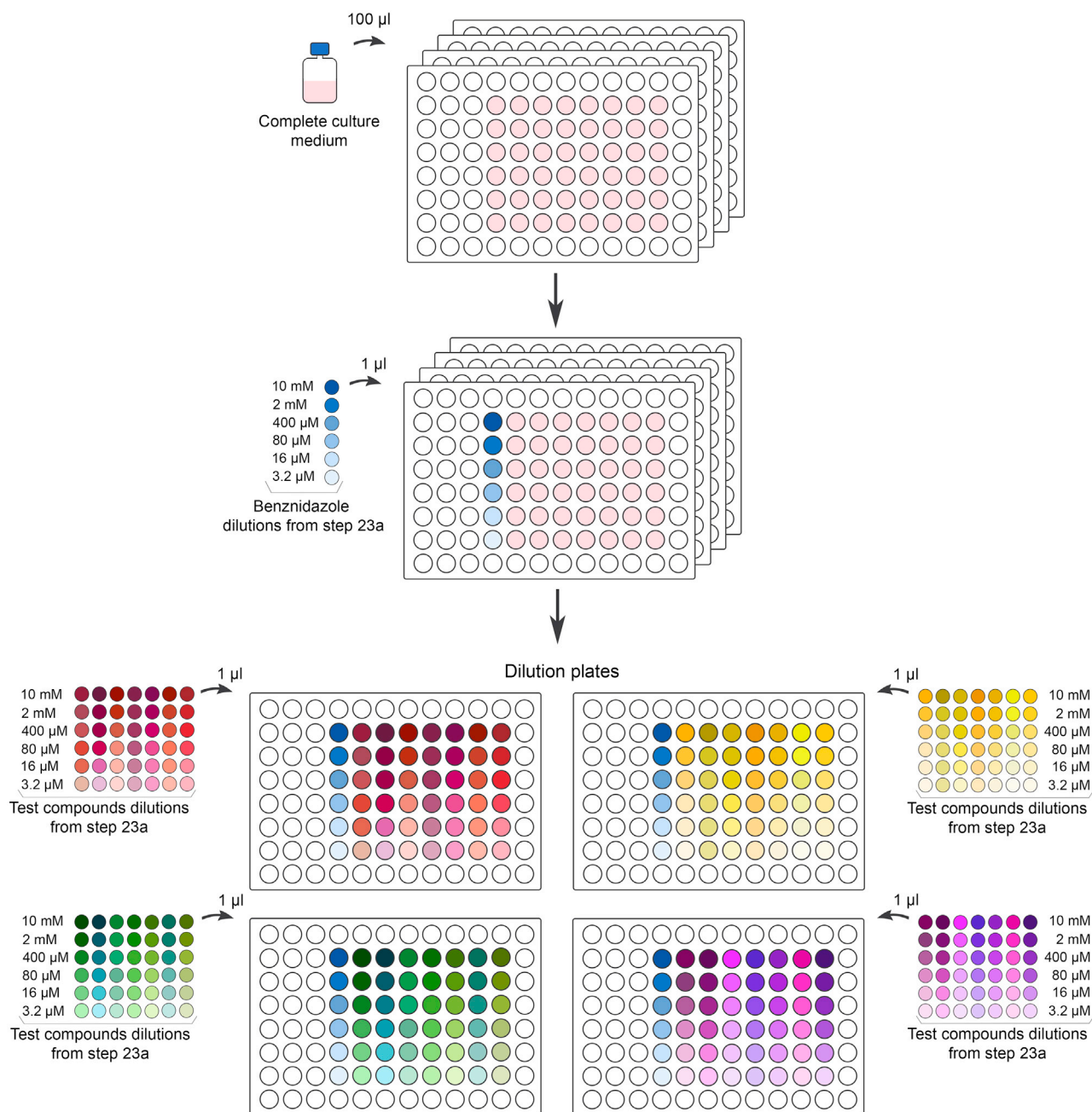


Figure 8. Scheme of compound dilution in complete culture medium

28. Discard and rinse with 150 μ L of DPBS.
29. Add 100 μ L of cell dye solution to each well, incubate for 15 min at 25°C and read on an Operetta System.

Note: At this stage the plates can be stored at 4°C for several days until reading. We analyzed plates stored for 3 weeks just checking before for crystal formations.

△ CRITICAL: If there is crystal formation in the bottom of the plates due to DAPI/Evans blue aggregation, wash the plates 3 times with PBS prior to image acquisition. This can happen

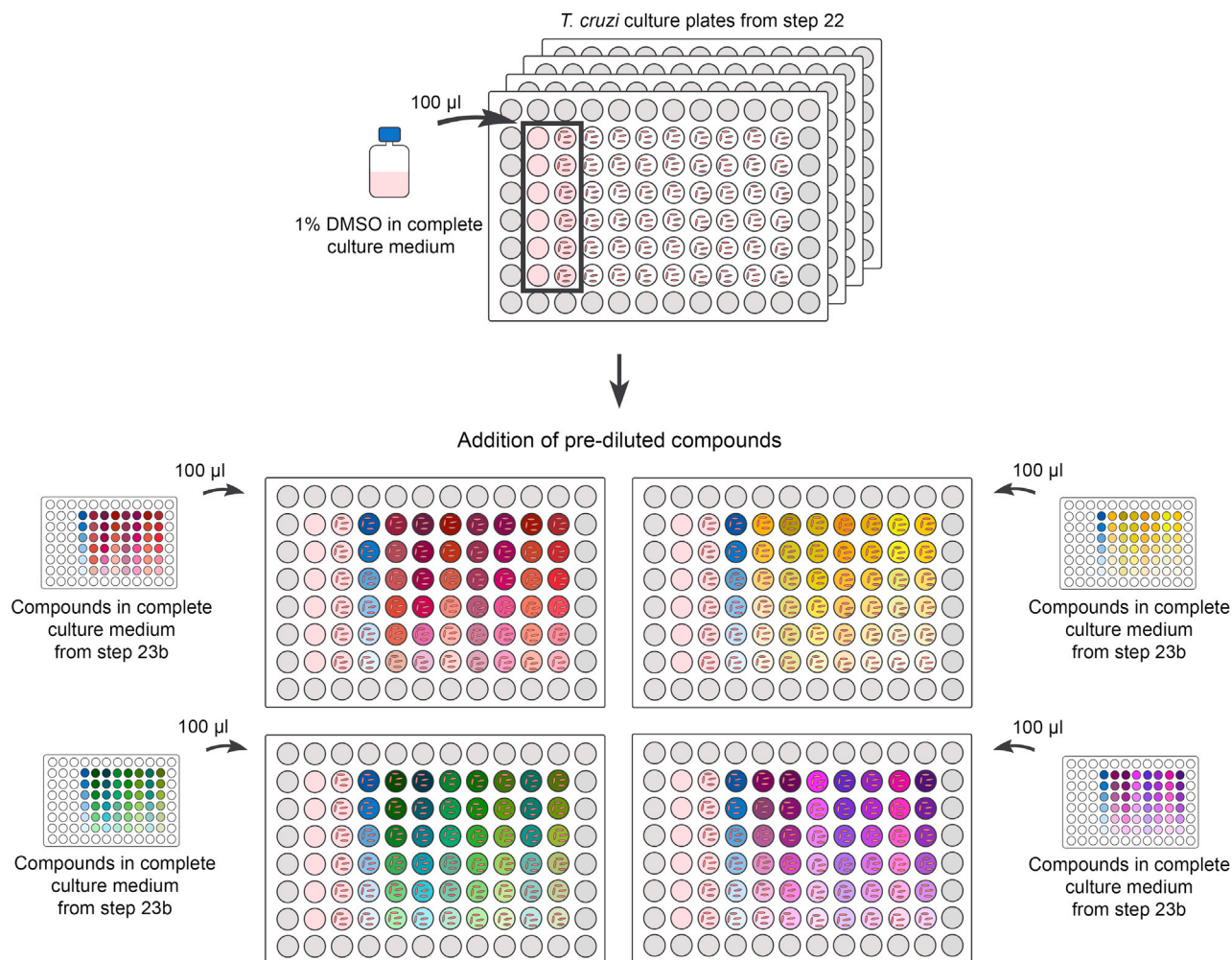


Figure 9. Display of a typical cell infection assay including non-infected and untreated controls, reference drug benznidazole and test compounds

mainly when using a low-quality paraformaldehyde and storage for longer periods (more than 3 weeks).

Proceed to the section “[processing of cell infection images using the Operetta System](#)” for data analysis.

Compound testing in cell infection assays – Phase II: Intracellular *T. cruzi* inhibition assay to determine IC₅₀, CC₅₀ and selectivity index (SI) and selection of candidates for *in vivo* testing

⌚ Timing: 10 days

In this section, the compounds selected from phase I are tested with replicates, in a wider concentration range and, if needed different treatment times, in order to obtain consistent data for reliable IC₅₀, CC₅₀ and selectivity index calculation with statistical support, allowing for appropriate estimation of compound efficiency against intracellular *T. cruzi* (Figure 11).

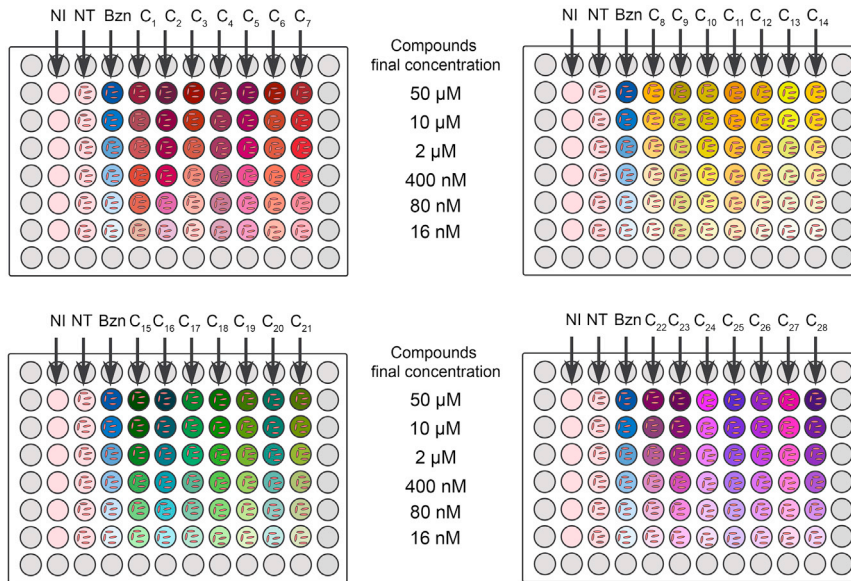


Figure 10. Outline of a 28-compound assay in four culture plates including non-infected and untreated controls, reference drug benznidazole and the 28 compounds

NI: non infected Vero cells; NT: untreated infected Vero cells; Bzn: reference drug benznidazole in infected Vero cells and C1 – C28: 28 different compounds in wells with infected Vero cells.

△ CRITICAL: Vero cells will be used throughout the whole procedure, so it is required always to keep them in culture (see [maintaining Vero cells culture](#)).

Note: In this section, the volume of cell and parasite are calculated for testing 3 different compounds using 10 different concentrations in quadruplicates for each compound concentration and 3 treatment times making a total of 9 plates, but adjustments can be made according to specific requirements. This arrangement allows for testing one compound per 96-well plate in parallel with untreated controls and non-infected cells on the same plate to reduce variation of culture conditions and experimental manipulations.

Day 1:

30. Thaw two frozen vials containing each 5×10^6 cell-derived trypomastigotes for amplification and obtention of fresh infective trypomastigotes.
 - a. Thaw rapidly by gentle agitation in a 37°C water bath and remove just before the content is fully thawed.

Note: To reduce the possibility of contamination, keep the O-ring and cap above the water.

- b. Transfer the trypomastigotes to a centrifuge tube containing 8 mL of complete culture medium and centrifuge at $3,000 \times g$ for 20 min at 12°C .
 - c. Discard the supernatant.
 - d. Suspend the trypomastigotes in 2 mL of complete culture medium.
31. Mix 1×10^6 Vero cells (see [preparing Vero cells for infection experiments](#)) with the metacyclic trypomastigotes recovered in the previous step (1×10^7 parasites).
32. Divide the mix of Vero and parasites cells in two 75 cm^2 culture flask, complete the volume of each to 10 mL with complete culture medium and incubate for 4 h at 37°C in a humid 5% CO_2 environment.

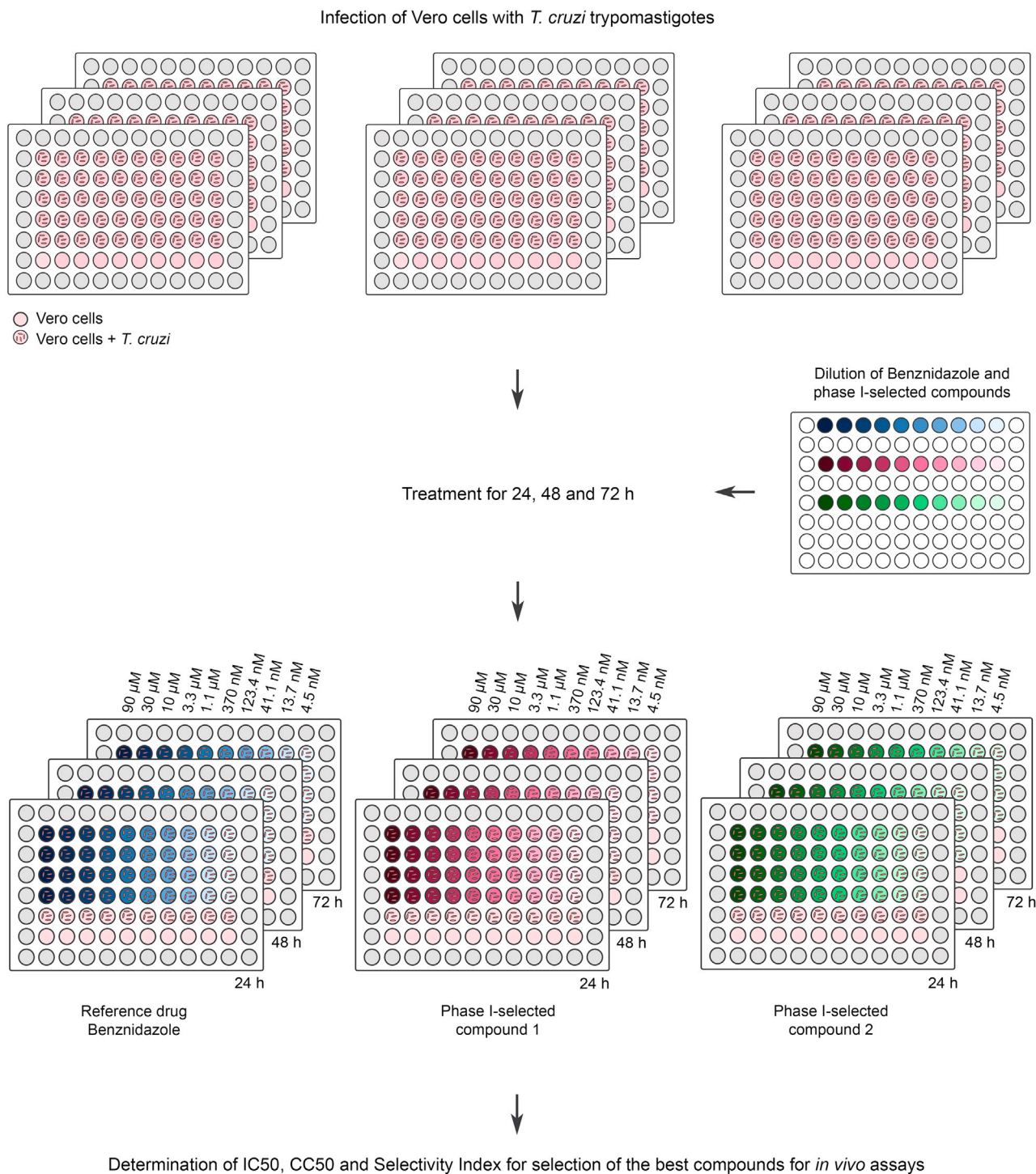


Figure 11. Overview of compound testing – Phase II

Plate distribution for each treatment. Positioning of the 10 diluted compound concentrations, expressed as μM or nM. 24 h, 48 h and 72 h: treatment time.

33. After 4 h of infection, discard the medium and rinse 2 times with 10 mL of DPBS to remove all the parasites that have not invaded the cells.
34. Add 10 mL of complete culture medium to each flask and incubate at 37°C in a humid 5% CO₂ environment.

Day 3:

35. In parallel, maintain a Vero cell culture for next steps. Inoculate 3.5×10^5 Vero cells in 10 mL of complete culture medium in a 75 cm² culture flask (see [maintaining Vero cells culture](#) and [Obtaining Vero cells for infection experiments](#)).
 - a. Incubate at 37°C in a humid 5% CO₂ environment.

Day 5:

36. At late afternoon, discard the medium of infected cells (from step 34) and rinse 2 times with 10 mL of DPBS to remove all the parasites that are already released, aiming to minimize new cycle of infections.
37. Add 10 mL of complete culture medium to each flask and incubate at 37°C in a humid 5% CO₂ environment.

Day 6:

38. In the morning, collect the released trypomastigotes by transferring the supernatant of both flasks to a centrifuge tube.
 - a. Recover the cell-derived trypomastigotes from the supernatant by centrifugation at $3,000 \times g$ for 20 min at 12°C.
 - i. Suspend parasites in 2 mL of complete culture medium and store at 4°C until the time of use.

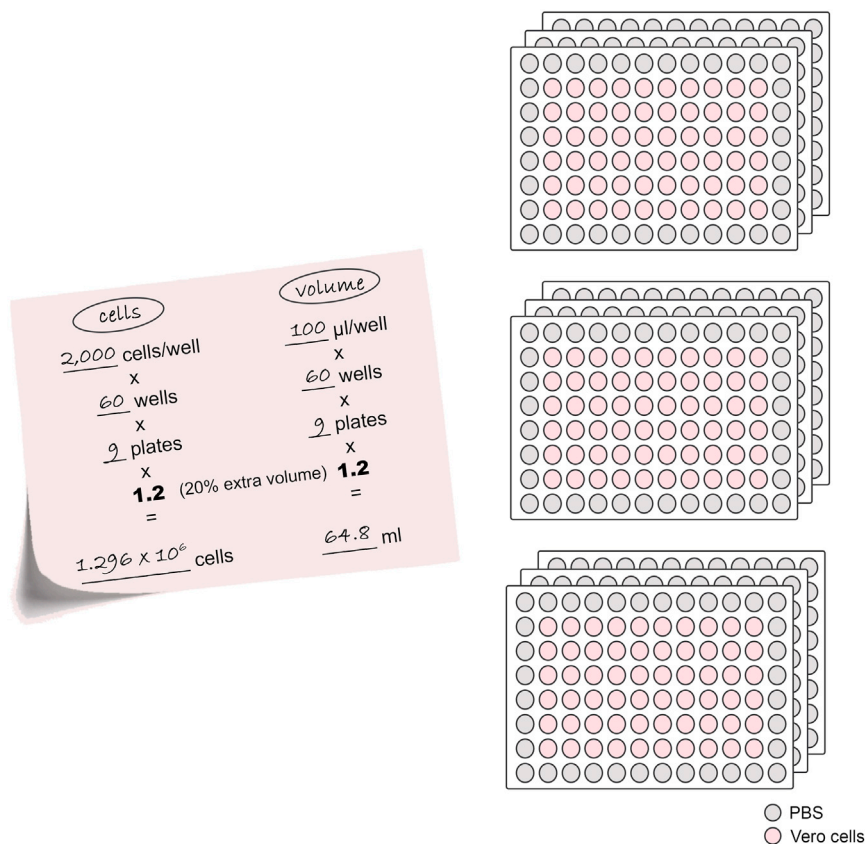


Figure 12. Plating of Vero cells for deep investigation assay



Figure 13. Outline of the plates used for refinement of compound activity in cell infection assays

- b. Add 10 mL of complete culture medium to the cell layer in each culture flask and incubate at 37°C in a humid 5% CO₂ environment.
39. Still in the morning, using the cells seeded at step 35 (see [preparing Vero cells for infection experiments](#)), dilute the appropriate quantity of Vero cells in complete culture medium to have 2×10^4 cells/mL (Figure 12).

Note: To ensure that the volume will be sufficient until the end of pipetting, it is recommended to make the calculations with an extra volume. In this work, we used a factor of 1.2.

- a. QUANTITY OF CELLS: $2,000$ cells per well \times 60 wells \times 9 plates \times 1.2 (20% extra volume) = 1.296×10^6 cells.
- b. TOTAL VOLUME: 100 μ L/well \times 60 wells \times 9 plates \times 1.2 (20% extra volume) = 64.8 mL.
40. Using a multichannel pipette, transfer 100 μ L of Vero cells (2×10^4 cells/mL) to each well of nine 96-wells culture plates as indicated in Figure 12. Incubate at 37°C in a humid 5% CO₂ environment.

Note: Do not use the wells at the border of the plate but do not leave them empty.

- a. Add 100 μ L of PBS at cell-free wells.
41. In the afternoon, recover the cell-derived trypomastigotes from the supernatant of both flasks from step 38b by centrifugation at $3,000 \times g$ for 20 min at 12°C. Suspend the trypomastigotes in 2 mL of complete culture medium.
42. Count the trypomastigotes from both parasite suspensions (“morning” from step 38a and “afternoon” from step 41) using a hemocytometer and a light microscope.

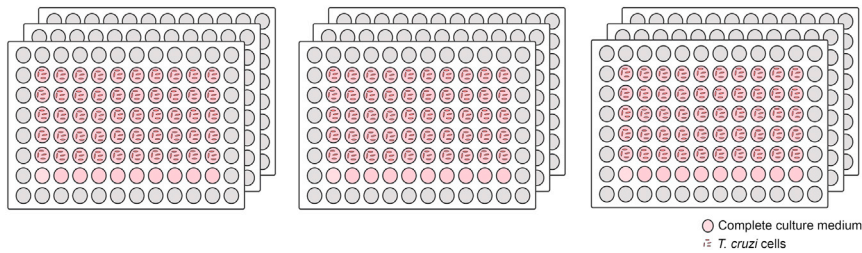


Figure 14. Scheme of the plates after washing and addition of complete culture medium

- a. Verify if the quantity of trypomastigotes recovered from step 41 ("afternoon" parasites) reaches the total needed in the next step. If not, combine with the trypomastigotes recovered from step 38a ("morning").

△ **CRITICAL:** If the total quantity is not enough for next step, abort the experiment and restart from step 30 (see also [problem 2](#)).

43. To infect Vero cells with a MOI of 20, dilute the appropriate quantity of trypomastigotes in complete culture medium so to have a concentration of 4×10^5 parasites/mL ([Figure 13](#)).
 - a. QUANTITY OF PARASITES: 20 (MOI) \times $2,000$ cells per well \times 50 wells \times 9 plates \times 1.2 safety margin = 2.16×10^7 parasites.
 - b. TOTAL VOLUME: $100 \mu\text{L/well} \times 50$ wells \times 9 plates \times 1.2 safety margin = 54 mL.
44. Using a multichannel pipette, transfer $100 \mu\text{L}$ of trypomastigotes (4×10^5 parasites/mL) to five rows of each of the nine 96-wells culture plates as indicated in [Figure 13](#).
45. Add $100 \mu\text{L}$ of complete culture medium to non-infected control wells.
46. Incubate the culture plates for 16 h–20 h at 37°C in a humid 5% CO_2 environment.

Day 7:

47. After 16 h of infection, carefully discard the supernatant by aspirating with a pipette. Rinse each well with $150 \mu\text{L}$ of DPBS to remove all the parasites that have not invaded the cells.

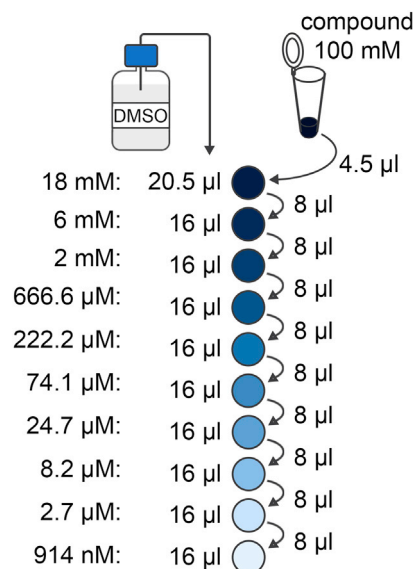


Figure 15. Pre-dilution of compounds

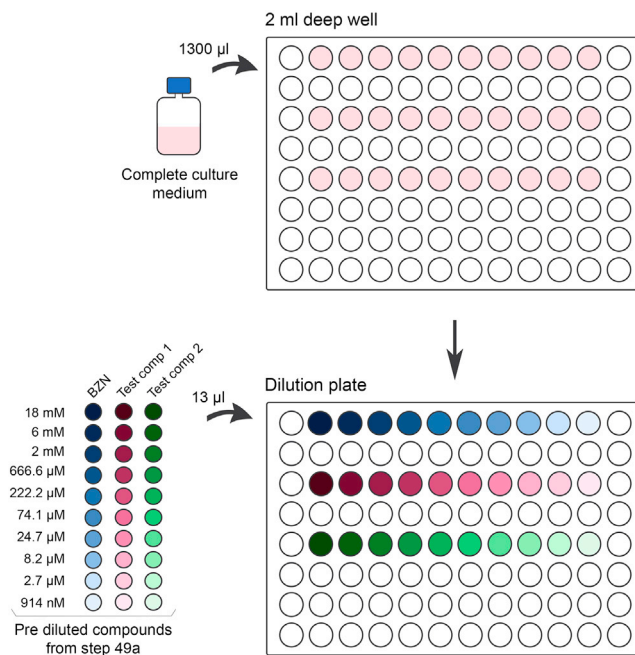


Figure 16. Scheme of compound dilution in complete culture medium

△ **CRITICAL:** Dispense the liquid by touching the tip to the side of the well and taking care not to touch the bottom. Pipette slowly so as not to disturb the cell layer (Figure 5).

48. Carefully remove all the remaining DPBS. Add 100 µL of complete culture medium to each cell-containing well (Figure 14) and incubate at 37°C in a humid 5% CO₂ environment.
49. Prepare the compounds for treatment:
 - a. Using 0.2 mL tubes, dilute the compounds in DMSO so to have a 10-fold dilution curve ranging from 18 mM to 914 nM as indicated in Figure 15.
 - b. Distribute 1,300 µL of complete culture medium (100 µL per well × ((number of replicates per plate) × (number of plates) + 1))) to a 2 mL deep 96-wells culture plates add 13 µL of the appropriate compound dilution (1 µL per well × ((number of replicates per plate) × (number of plates) + 1))) (Figure 16). Mix thoroughly.
50. Treat cells (from step 48) as indicated above and in Figure 17:
 - a. to the non-infected and untreated control wells, add 100 µL of 1% DMSO in complete culture medium.
 - b. to the compound test wells, add 100 µL of the mixture prepared in the step 49b.
51. Incubate all the 96-wells culture plates (Figure 18) at 37°C in a humid 5% CO₂ environment for the correspondent times.

Day 8:

52. After 24 h of treatment, prepare the set of 24 h-treated cells to acquire the images on an Operetta System (PerkinElmer) or other high-content imaging system.
53. Fix and stain the cells:
 - a. Carefully discard the supernatant by aspirating with a pipette.
 - b. Rinse each well with 150 µL of DPBS.
 - c. Add 100 µL of 4% paraformaldehyde and incubate for 15 min at 20°C–25°C.
 - d. Discard and rinse with 150 µL of DPBS.
 - e. Add 100 µL of cell dye solution to each well.
 - f. Store at 4°C for several days until reading at Operetta System.

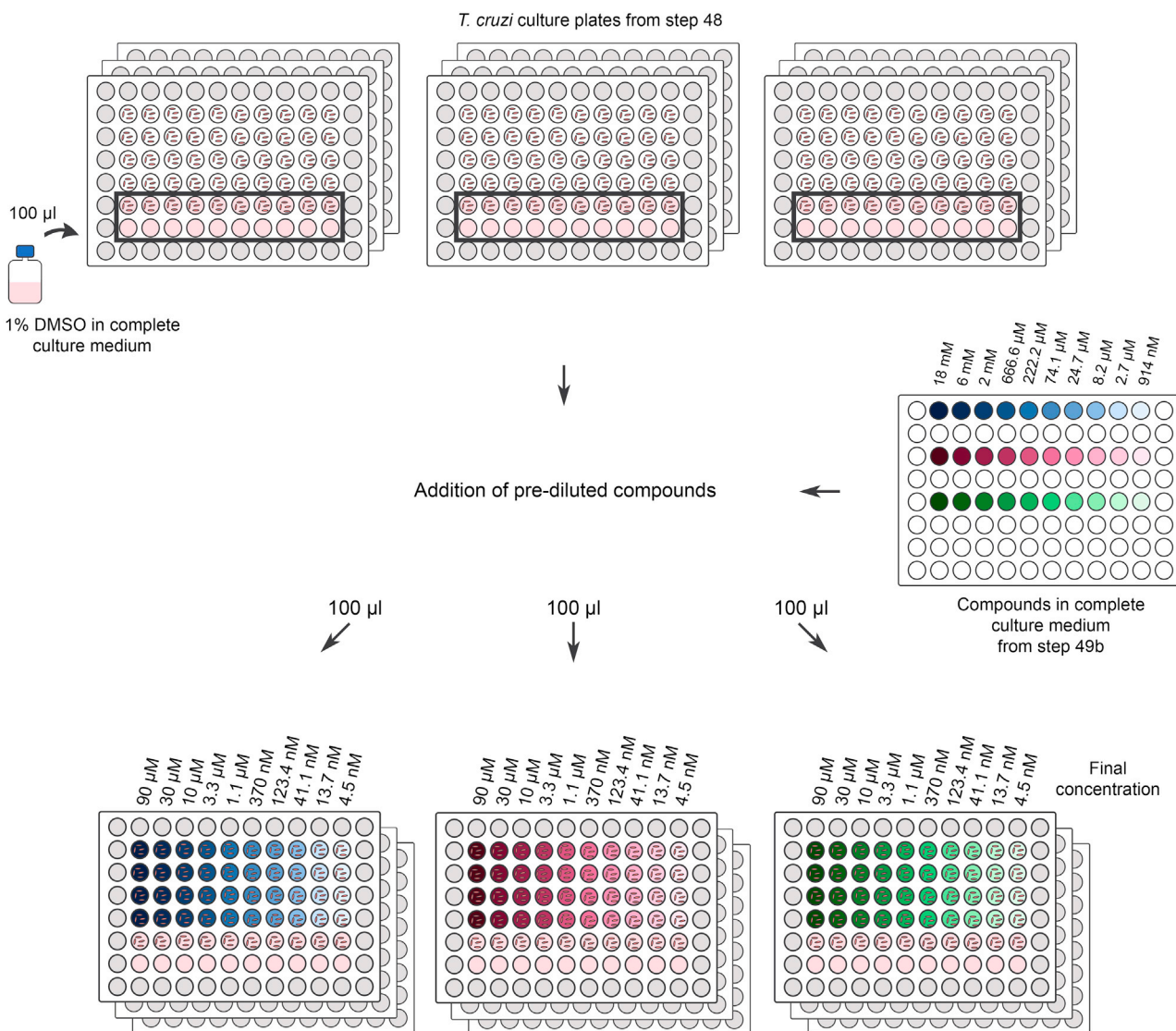


Figure 17. Treatment of cells including non-infected and untreated controls, reference drug benznidazole and pre-selected compounds also indicating the final compound concentration tested in each well

Day 9:

54. After 48 h of treatment, prepare the set of 48 h-treated cells to acquire the images on an Operetta System.
55. Fix and stain the cells as indicated in step 53.

Day 10:

56. After 72 h of treatment, prepare the set of 72 h-treated cells for reading on an Operetta System.
57. Fix and stain the cells as indicated in step 53.

Note: The work of this section was done by dividing some tasks between two people.

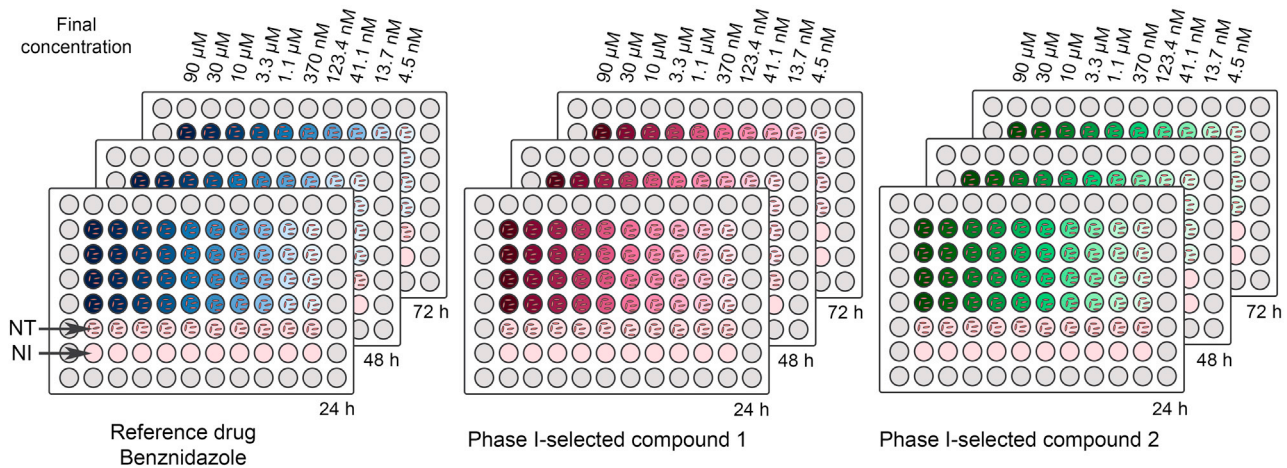


Figure 18. Complete set of plates including non-infected and untreated controls and quadruplicates of compound dilutions
NI: non infected Vero cells; NT: untreated infected Vero cells; 24 h, 48 h and 72 h: treatment time.

Note: The staining of different host cell structures can also be done in parallel to provide rough insights on the mechanism of action.

Proceed to section “[processing of cell infection images using the Operetta System](#)” for data analysis.

T. cruzi inhibition assay in mouse infection model

⌚ Timing: 10 days

In this step, the compounds will be tested and compared in parallel with the reference drug benznidazole using animal model (Figure 19).

⚠ **CRITICAL:** Before starting this section, permission for the use of animals should have already been obtained from an Animal Experimentation Ethics Committee and the mice should be available. See also [before you begin Section - obtaining mice for infection and compound testing](#).

Note: In this work, all the animal experiments were carried out at the Animal Facility of FIOCRUZ ICC Paraná (Laboratory of Animal Models – LACEA) which is responsible to provide housing and husbandry. Specific Pathogens Free (SPF) animals, obtained from certified matrices imported from the Charles River laboratory – USA, are kept under aseptic conditions. All animals – individually identified and grouped according to their respective treatment, with a maximum of 5 animals per cage – are kept inside sterile ventilated racks and are always handled within laminar flows ensuring their sanitary integrity.

58. Thaw a frozen vial containing 5×10^6 cell-derived trypomastigotes for amplification and obtainment of fresh infective trypomastigotes.
 - a. Thaw rapidly by gentle agitation in a 37°C water bath and remove just before the content is fully thawed.

Note: To reduce the possibility of contamination, keep the O-ring and cap above the water.

- b. Transfer the trypomastigotes to a centrifuge tube containing 9 mL of complete culture medium.

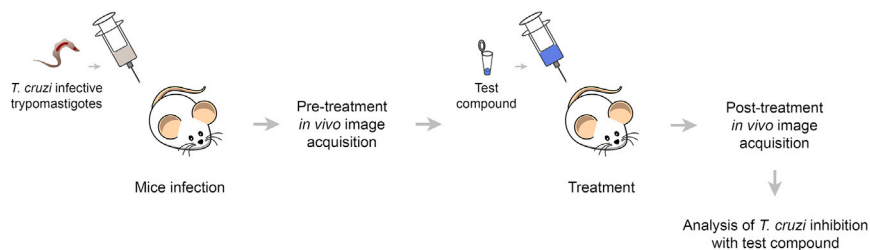


Figure 19. Overview of *T. cruzi* inhibition assay in mouse infection model

- c. Centrifuge at $3,000 \times g$ for 20 min at 12°C .
 - d. Discard the supernatant.
 - e. Suspend the parasites in 1 mL of complete culture medium.
59. Infect 5×10^5 Vero cells (see [Obtaining Vero cells for infection experiments](#)) with thawed trypomastigotes (5×10^6 parasites) as described in step 3.

Infection day:

60. Recover cell-derived trypomastigote from the previous step and suspend parasites in DPBS to obtain 10^6 cells/mL.
- a. Prepare an appropriate volume for all inoculations: final volume = $(100 \mu\text{L per mouse}) \times [(\text{number of mice per condition}) \times (\text{number of conditions: untreated, reference drug benznidazole and test compound}) + 2^*]$.

Note: * 2 extra doses for syringe dead volume.

Note: *T. cruzi* strains present distinct virulence and require different amounts of parasites to cause an adequate acute infection and subsequent detection. The indicated parasite load of 10^5 parasites/mouse was standardized for *T. cruzi* Dm28c strain.

61. Inoculate $100 \mu\text{L}$ of infective parasites intraperitoneally in each mouse ([Figure 20](#)).

△ CRITICAL: Animal handling and inoculations should be performed exclusively by trained personnel with prior expertise.

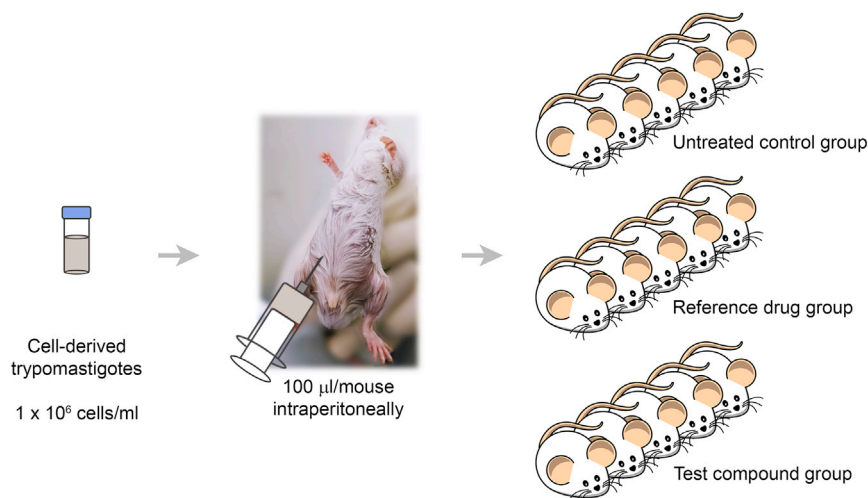


Figure 20. Infection of mice with *T. cruzi*

Day 3 post infection:

62. Acquire pre-treatment mice images.
 - a. Prepare D-luciferin for inoculations considering the recommended concentration of 150 mg/kg:
 - i. Weight the mice and calculate the average weight.
 - ii. Weight D-luciferin for all inoculations: total quantity = (average of mice weight in kg) × [(number of mice per condition) × (number of conditions (untreated, reference drug benznidazole and test compound)) + 2*] × 150 mg/kg.
 - iii. Dilute the D-luciferin in DPBS in an appropriate volume for all inoculations: final volume = 100 µL per mouse × [(number of mice per condition) × (number of conditions (untreated, reference drug benznidazole and test compound)) + 2*].

Note: * 2 extra doses for syringe dead volume.

- b. Administer 100 µL of D-luciferin in the intraperitoneal cavity of the mice and wait for 8 min.
- c. Anesthetize the mice.
 - i. Adjust the oxygen flowmeter to 0.4–0.8 l/min in a box chamber and isoflurane vaporizer to 2.5% and wait for 2 min.
 - ii. Place the animals in the nosecone of the image acquisition equipment, maintaining the anesthetizing gas and oxygen during the entire process.
- d. Acquire images of pre-treatment mice on ventral and dorsal positions using an IVIS Spectrum CT scanner (other luminescence living image scanners could be used) within 10–15 min after D-luciferin inoculation.
 - i. Take both auto and 30 s timed acquisitions.
 - ii. Ensure that the positioning of animals is reproduced in each acquisition, with the animals being placed in the same order.

△ **CRITICAL:** The time interval between D-luciferin inoculation and image acquisition is critical for proper comparison between groups. Ensure that the delayed time for anesthetizing and positioning animals on the IVIS is reproducible to minimize processing differences between groups. This time interval should be previously tested to determine the peak signal intensity and the time window. We found no significant difference in the 10–15 min interval.

Note: If no signal was detected at this stage, please refer to [problem 8](#): in [troubleshooting session](#).

Note: Images are processed using the Living Image software. See description on Section [quantification and statistical analysis - processing of images using the Living Image Software](#).

Day 4 post infection:

63. Dilute compounds just prior to administration.
 - a. Solubilize the weighted and aliquoted compounds (from Section: [preparing compounds and reference drug for mice treatment](#)) in DMSO so the final concentration is 7% and mix well.
 - b. Add 50% HP-β-cyclodextrin prepared in DPBS so the final concentration is 40% and mix well.
 - c. Complete volume with DPBS.
64. Treat each group of mice with intraperitoneal injection of 100 µL of the diluted compounds or vehicle for the control group ([Figure 21](#)).

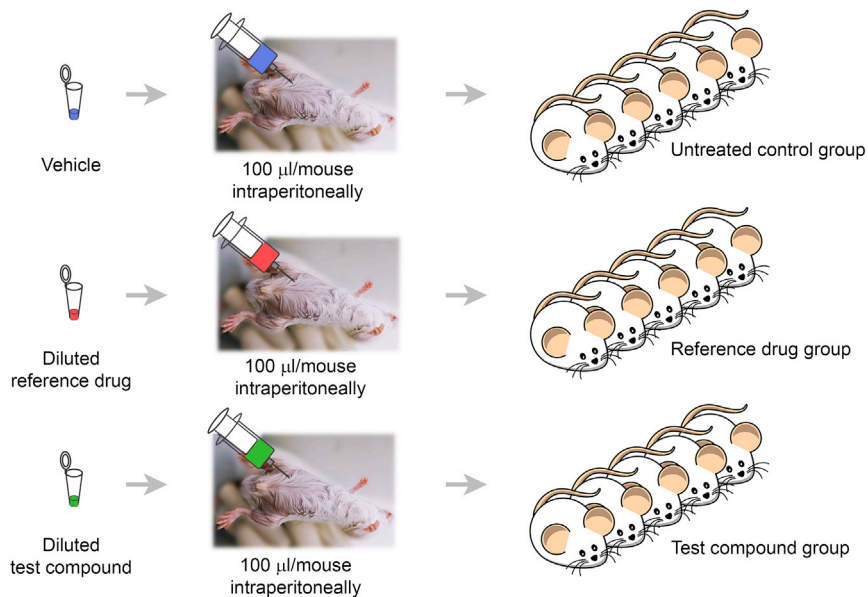


Figure 21. Treatment of mice

Day 5 post infection:

65. Repeat the treatment as indicated in steps 63 and 64 in alternate side of the abdominal cavity to minimize lesion formation.

Day 6 post infection:

66. Repeat the treatment as indicated in steps 63 and 64.

Day 7 post infection:

67. Repeat the treatment as indicated in steps 63 and 64 in alternate side of the abdominal cavity.
68. Acquire images for parasite load determination by luminescence quantification as indicated in step 62.

Day 8 post infection:

69. Repeat the treatment as indicated in steps 63 and 64.

Day 9 post infection:

70. Repeat the treatment as indicated in steps 63 and 64 in alternate side of the abdominal cavity.
71. Acquire images for parasite load determination by luminescence quantification as indicated in step 62.

Note: If the luminescence intensity signal was higher in the treated group than in the control group, see [troubleshooting - problem 9](#).

EXPECTED OUTCOMES

The integrative platform, which includes both cell and animal infection models, provides a reliable pipeline for selecting compounds with higher potential to advance to clinical trials.

The Phase I screening, where a large number of compounds can be tested in a shorter time, is expected to speed up the process of excluding the compounds that are non-active against *T. cruzi* or toxic to the host cells based on the levels of intracellular *T. cruzi* inhibition and host cell toxicity.

The compounds pre-selected in Phase I are then tested in the Phase II intracellular *T. cruzi* inhibition assays, which includes a wider concentration range, a greater number of replicates and longer treatment times. Phase II is expected to reveal the non-toxic compounds with higher inhibition activity against intracellular *T. cruzi* with their respective IC_{50} , CC_{50} and selectivity index, which are the parameters required for a decision about which compounds should be tested in animal models.

In the final step, the compounds selected in the cell infection assays are tested in an animal model and their efficiency compared to the reference drug benznidazole. This step is expected to reveal the compound candidates with the appropriate features to proceed to the clinical trial phase.

QUANTIFICATION AND STATISTICAL ANALYSIS

Processing of cell infection images using the Operetta System

The power of high content imaging systems relies on automation but also on the development of specific software that may process images and include built-in adjustable analysis that provide computation of intended data for the final calculations.

Note: The following topics will focus on guidelines for parameter adjustments that may be required by all types of high content imaging systems. However, here they refer to the Harmony software coupled to the Operetta System and may contain specificities of this platform. Users of other systems should address the corresponding manual.

Alternatives: Although not tested, other freely available image analysis programs like ImageJ and CellProfiler can be used as well.

Acquire the images on a high content imaging system using the adequate parameter settings:

- Plate: select type and brand of plates used for specific dimensioning by the automated image acquisition unit. Regular clear plates are adequate for this type of assay.
- Objectives: a dry 20× objective (N.A. 0.4) is sufficient for detecting the intracellular parasite nucleus and kinetoplast. Moreover, it provides a larger field including more cells in each field of view.
- Channels: select the channels for each stain used in the assay – for DAPI detection of nuclear staining use excitation at 355–385 nm and emission at 430–500 nm, and for cytoplasm stained with Evans' blue use the parameters for Alexa 633, with excitation at 615–645 nm and emission at 655–760 nm.
- Plane: acquiring images in a single adjusted focal plane is sufficient when using Vero cells due to their flatness when adhered. Consequently, amastigotes are accommodated in the same focal plane in the Vero cell cytoplasm. Determine the best focus using the Stack function by testing the acquisition of 5 images with a distance of 1 μm from each other in a single selected field. Do this procedure for each channel, as nucleus and cytoplasm may require distinct planes for the best focus. The decision of the Height parameter should be included in the Channel Selection. Remove the Stack parameters when scanning the full plate.

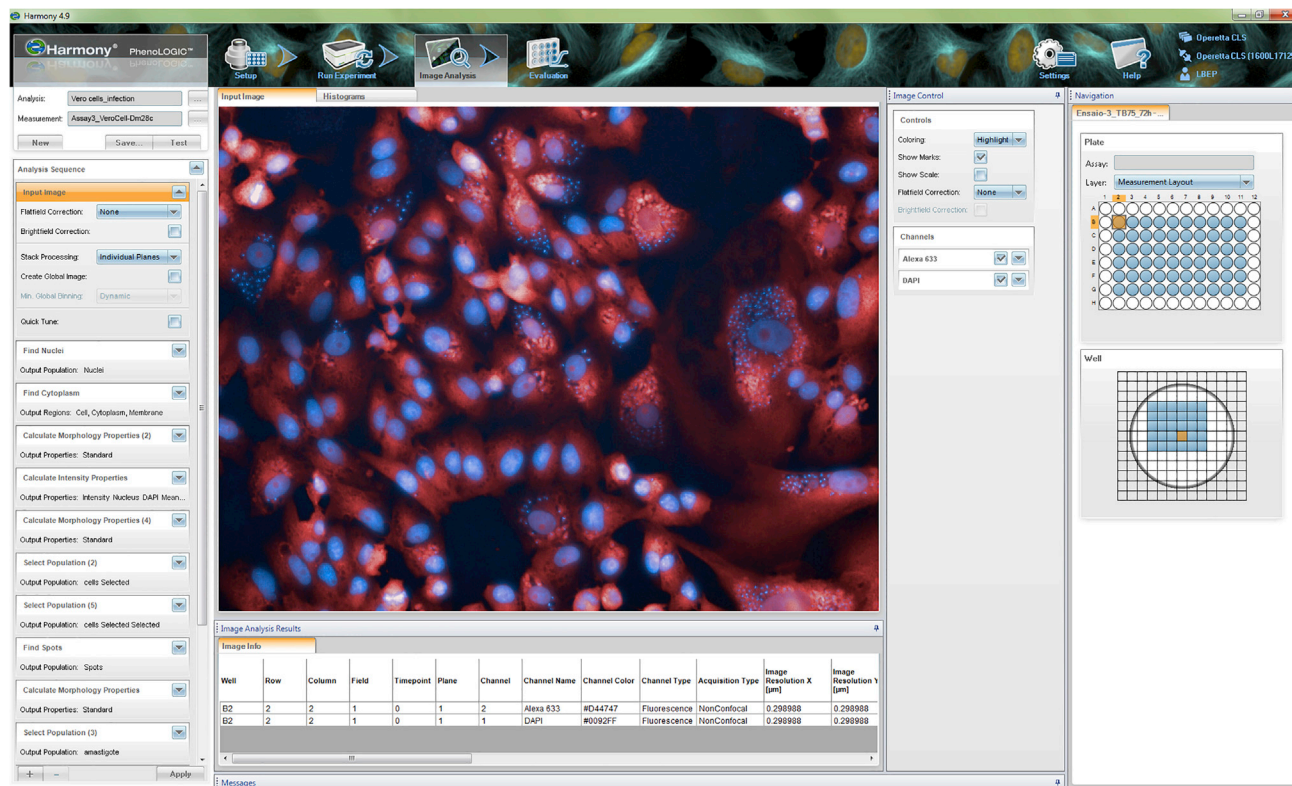


Figure 22. Screen visualization of the Image Analysis tool of the Harmony software

On the left, the Analysis Sequence visualization with the required building blocks. In the center, the image of the selected field for evaluation of the parameters chosen. On the right, navigation controls.

- Fields: selecting 20–25 central fields on each well should be enough for analyzing around 3,000 cells, depending on time elapsed, when using the 20× objective.
- Image specifications: the Operetta system is equipped with a 16 bit sCMOS camera, acquiring 4.7 Megapixel images (2160 × 2160) with 6.5 µm pixel size.

△ **CRITICAL:** Ensure that the selected fields capture the center of the wells in all positions of the plate.

The most important aspect of every image analysis is good image quality. For this, visual inspection of the images is required to include in the analysis only those with sufficient quality for segmentation.

Image analyses were performed essentially using the Harmony software by combining a series of pre-made building blocks with fine adjustments of specific parameters according to each image requirement.

The first step is image segmentation, which can be described as the identification of the primary objects of interest that will be further analyzed in subsequent steps. It involves breaking down the image into discrete objects. Below, the building blocks used for segmentation are described including some additional information about parameters' definitions.

△ **CRITICAL:** For all steps, choose the appropriate method by trial and error using the visual feedback display, aiming the best identification of the desired image segments, which in this case are the outline of the Vero cell cytoplasm, the Vero cell nucleus and the nucleus and kinetoplast of intracellular *T. cruzi* forms.

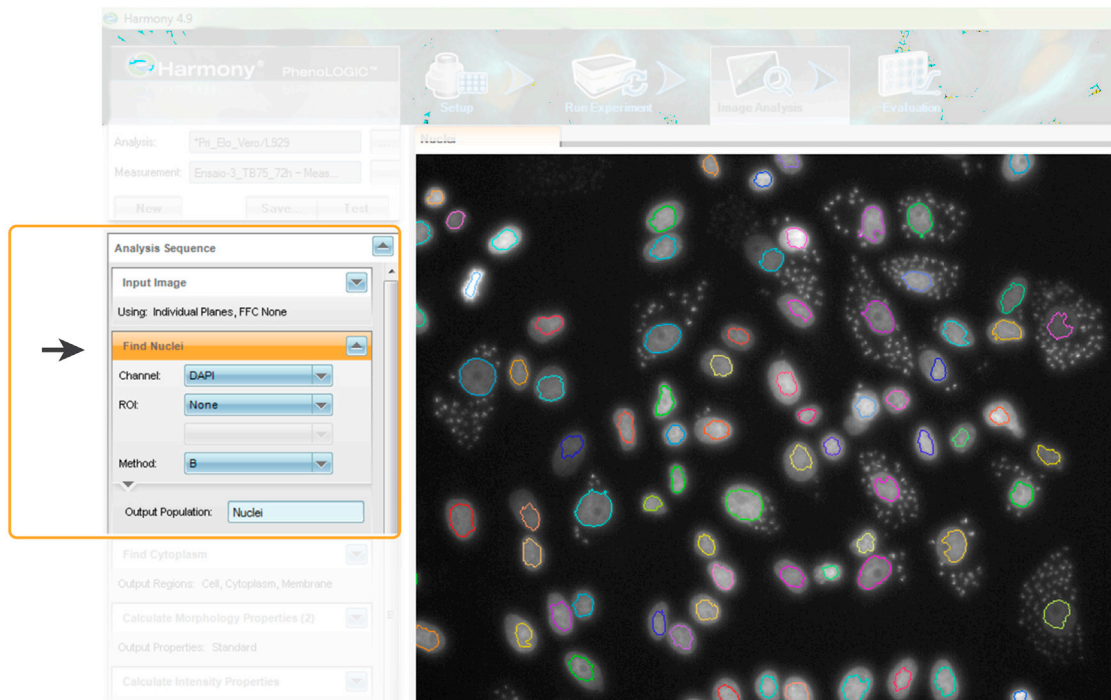


Figure 23. Screen visualization of parameters used for definition of Vero cells Nuclei population

1. Create an Analysis Sequence in the Image Analysis tool (Figure 22).
2. Add the building block Input Image and select the option to process individual planes.
3. Identify the Vero cells:
 - a. Add the building block Find nuclei (Figure 23).
 - i. select the DAPI channel,
 - ii. select the best built in method (choose A, B, C or M) to detect cell nucleus and adjust the parameters aiming to reduce the loss of significant objects while avoiding grouping of intracellular *T. cruzi* nuclei (Figure 24). This and further selection analysis may be easier by using the Tune Parameter command when right clicking over the intended parameter.

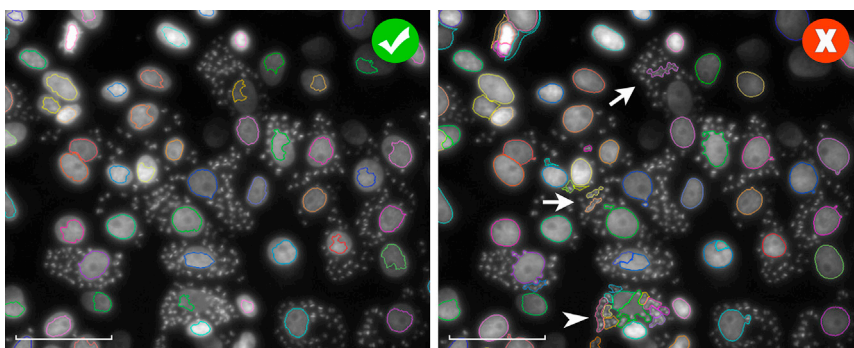


Figure 24. Comparison of methodologies used for selection of Vero cell nuclei

On the left, the method presented an adequate selection of objects and nuclear area, though with some size discrepancies. On the right, the method erroneously identifies grouped parasite nuclei as constituting a eukaryotic nucleus (indicated by arrows) and it also splits a single nucleus into one or more objects (indicated by an arrowhead). Each colored contour represents one individual event. Bar: 50 μ m.

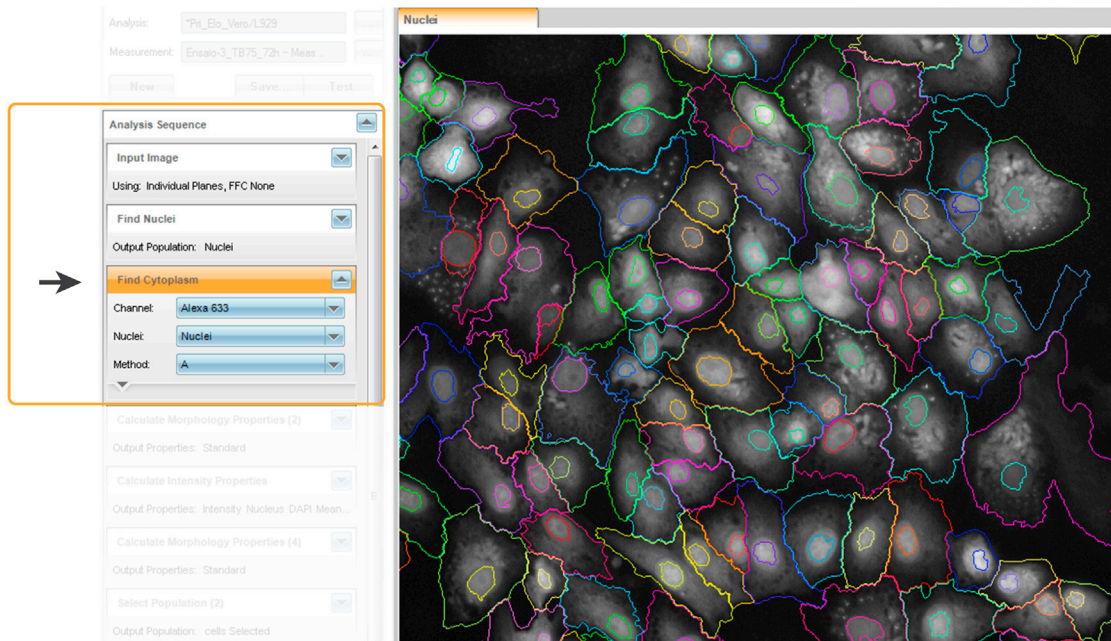


Figure 25. Screen visualization of parameters used in the definition of Vero cells cytoplasm population

- iii. it is possible to tune second level parameters in the method by expanding the options below the Method selection. Increase or decrease the Common Threshold to exclude dark objects or include dark nuclei, respectively. Increasing the lower limit of the Area of the detected nuclei may also aid to exclude small objects.
- iv. name the Output Population as *Nuclei*.
- b. Add the building block Find cytoplasm.
 - i. Select the Alexa 633 channel.
 - ii. Use previously detected *Nuclei* population to indicate which cells contain identified nucleus (Figure 25).
 - iii. Select the best fit Method for the cytoplasm determination. Evans' blue staining helps to delimit cell boundaries, which will comprise the amastigotes' search area. Ensure that the selected method includes an adequate span of each cell limit, not too restricted to the

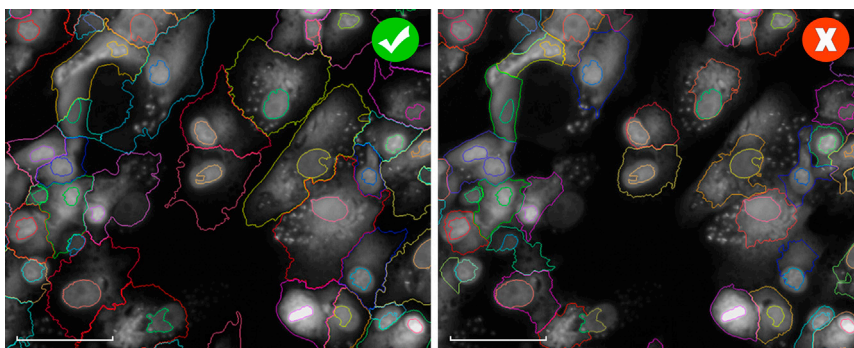


Figure 26. Example of two cytoplasm identification methodologies

On the left, the method boundaries obey full cytoplasm staining, while on the right the area identification is restricted to intensely stained areas, thus losing intracellular space. Each colored line delimits an individual cell. Nuclei are also shown in colors. Bar: 50 μ m.

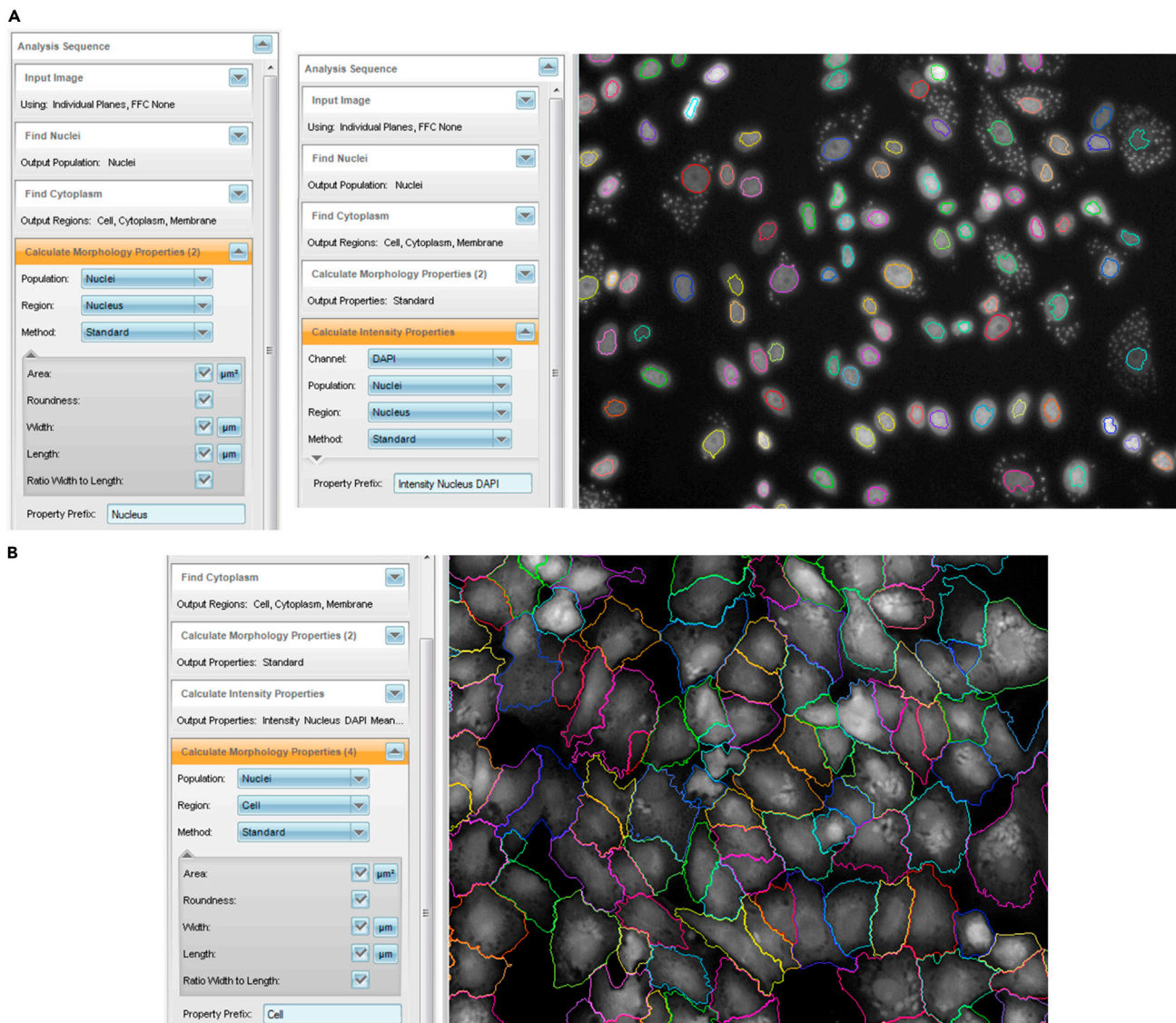


Figure 27. Screen visualization of parameters used to limit the cell population

(A) Settings used to better define Vero cells identification based on nuclear morphology (left) and nuclear intensity (right) properties. (B) Settings used to better define Vero cells identification based on cell morphology properties.

nucleus surrounding, but also not too wide to avoid including the extracellular space (Figure 26). Tune the Individual Threshold to improve boundary determination particularly in less populated areas on the edges of the wells.

4. Calculate properties that may be used to limit the cell population (Figure 27).
 - a. Add the building block Calculate Morphology Properties.
 - i. Select Population *Nuclei*.
 - ii. Select Region *Nucleus*.
 - iii. Select Method *Standard*.
 - iv. Expand the options menu and mark all the fields for Area, Roundness, Width, Length and Ratio Width to Length parameter calculations.
 - v. Name the Property Prefix as *Nucleus* (Figure 27A, left).
 - b. Add the building block Calculate Intensity Properties.

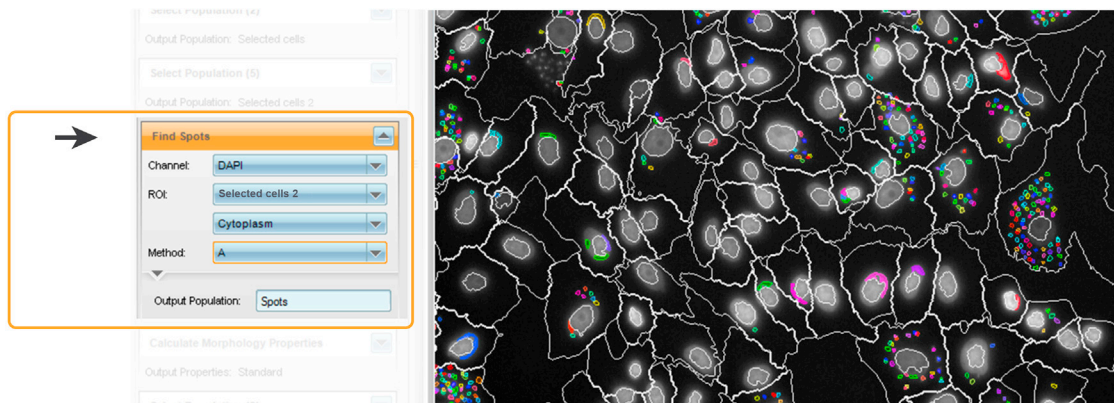


Figure 28. Screen visualization of parameters used to select amastigote populations

- i. Select Channel DAPI.
- ii. Select Population *Nuclei*.
- iii. Select Region Nucleus.
- iv. Select Method Standard.
- v. Expand the options menu and mark the field Mean.
- vi. Expand the second options menu and mark the fields Median, Sum, Maximum and Minimum.
- vii. Name the Property Prefix as *Intensity Nucleus DAPI* (Figure 27A, right).
- c. Add another building block Calculate Morphology Properties.
 - i. Select Population *Nuclei*.
 - ii. Select Region Cell.
 - iii. Select Method Standard.
 - iv. Expand the options menu and mark all the fields for Area, Roundness, Width, Length and Ratio Width to Length parameter calculations.
 - v. Name the Property Prefix as *Cell* (Figure 27B).
5. Delimit the cell population to exclude unintended objects or to select specific cells to create specific populations, e.g., by the size and shape of cells.
 - a. Add the building block **Select Population**. This step may be skipped if the cell selection in the last steps was accurate enough to avoid unintended objects or if no specific population selection is required.
 - i. Select Population *Nuclei*.
 - ii. Select Method Filter by Property.

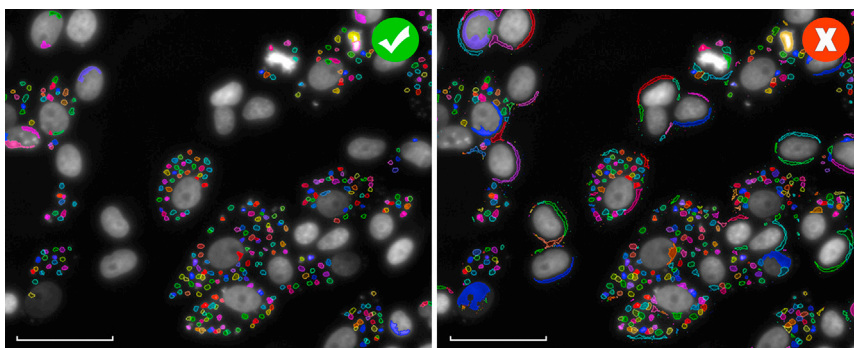


Figure 29. Comparison of methodologies used to identify spots in the cytoplasm

On the left, an adequate method which includes most of the observed *T. cruzi* amastigotes. On the right, a method too sensitive includes small points and nuclear artifacts as objects. Each individual object is distinguished by colored contour. Bar: 50 μ m.

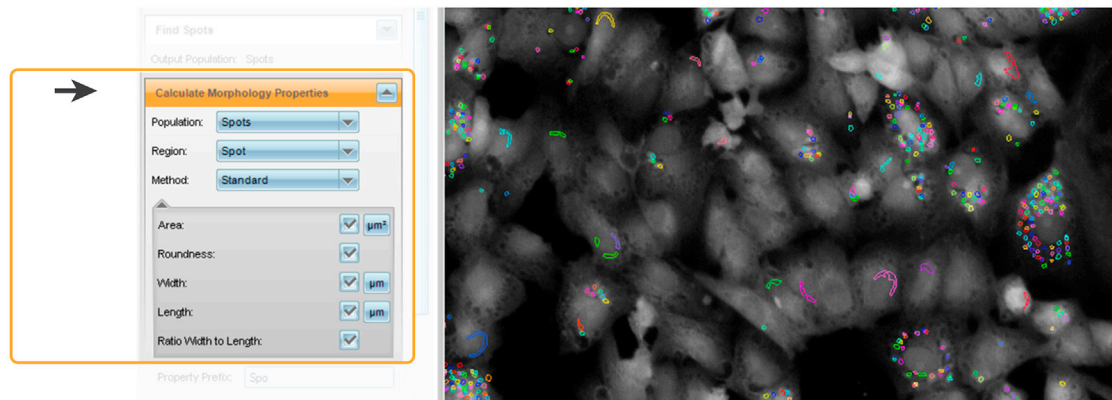


Figure 30. Screen visualization of parameters used to refine amastigote identification based on morphology properties

- iii. Expand the options menu and delimit the population by using the calculated properties in order to avoid unintended objects, e.g., delimit Nucleus Width by sizes below 50 μm . On the top of the visualization image, select the tabs Scatter Plots or Histograms to visualize calculated values observed in the selected field. To aid the analysis, select a single or a group of points on the Scatter Plots tab and look back to the image tab to evaluate the visualized cells and determine the best cutoffs to include real nuclei and to avoid spurious objects.
- iv. Name the Output Population as *Selected Cells* or by the specific filtered parameters.
- b. Add a building block **Select Population**. This step is required to remove cells at the border of the fields.
 - i. Select the Population *Selected Cells*.
 - ii. Select the Method Common Filters.
 - iii. Mark the option Remove Border Objects.
 - iv. Name the Output Population *Selected Cells 2*.
6. Identify the *T. cruzi* amastigotes.
 - a. Add the building block **Find Spots** (Figure 28).
 - i. Select the Channel DAPI.
 - ii. Select the image *Selected Cells 2* and the Cytoplasm region in the field ROI.
 - iii. Select the best Method of identification. Determine the selection of the kinetoplast DNA from amastigote cells, which is smaller and more intense than the parasite nuclei in DAPI staining. In some situations, both may be recognized as a single object, depending on the selected parameters.
 - iv. Expand the menu options and tune the parameters for spot identification inside the selected method. Each method includes a set of specific parameters to be adjusted. Use the Tune Parameter utility for a quicker visualization of changes by right clicking on the intended parameter.
 - v. Expand a second menu options and mark the Calculate Spot Properties.
 - vi. Name the Output Population as *Spots*.

△ CRITICAL: The first round of selection is intended to identify most of the parasites inside the cell, so caution should be taken to balance between missing events and excessive inclusion of cell artifacts (Figure 29).

- b. Add the building block **Calculate Morphology Properties**.
 - i. Select the Population *Spots*.
 - ii. Select the Region *Spot*.
 - iii. Select the Method *Standard*.

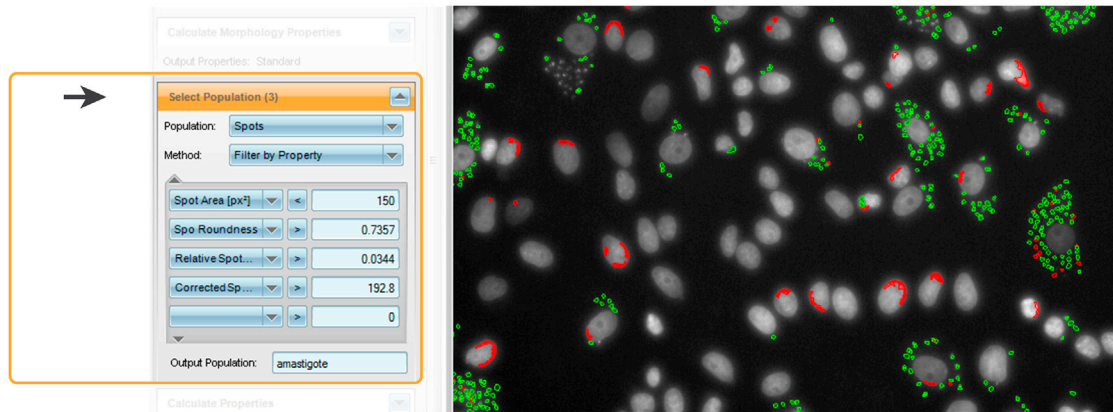


Figure 31. Screen visualization of parameters used to refine amastigote identification

- iv. Expand the options menu and mark the parameters Area, Roundness, Width, Length and Ratio Width to Length (Figure 30).
- c. Add the building block Select Population.
 - i. Select the Population Spots.
 - ii. Select the Method Filter by Property.
 - iii. Expand the options menu and include any calculated parameter that may be used to define the amastigote population, selecting with highest fidelity the parasite population inside each cell while avoiding the exclusion of correct objects. The parameters used were Spot Area, Spot Roundness, Relative Spot Intensity and Corrected Spot Intensity. Use the Scatter Plots tab and the Tune Parameter to aid with this selection.
 - iv. Name the Output Population as Amastigotes (Figure 31).
7. Identify infected cells.

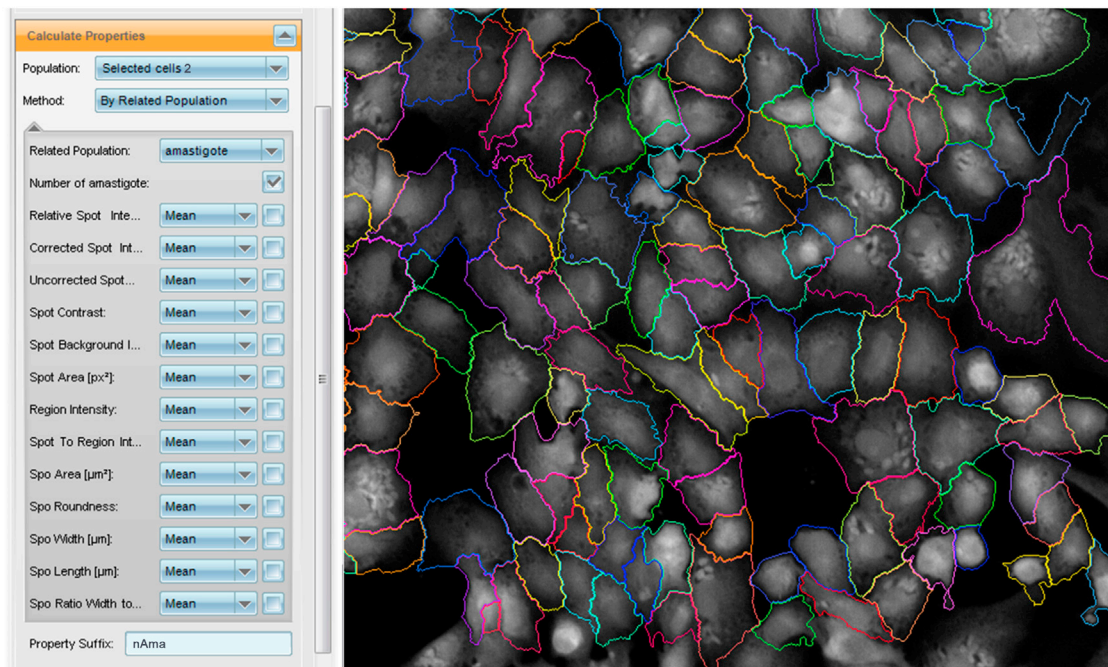


Figure 32. Screen visualization of parameters used to count the number of amastigotes inside the cells

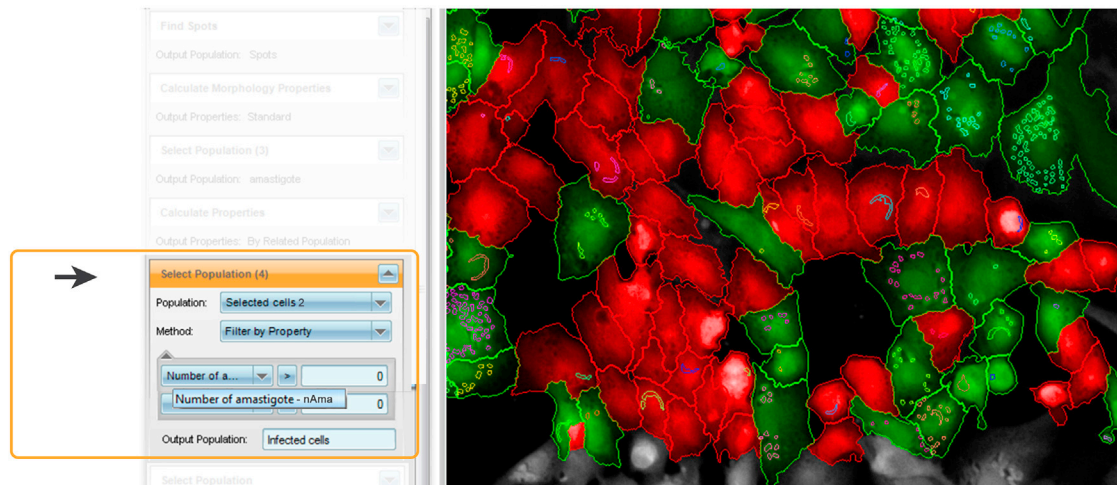


Figure 33. Screen visualization of parameters used to define infected cells

Green indicates infected cells, red uninfected cells and, gray cells that were not counted.

- a. Add the building block **Calculate Properties**. This step is required to count the number of amastigotes inside each cell.
 - i. Select the Population *Selected Cells 2*.
 - ii. Select the Method named *By Related Population*.
 - iii. Expand the options menu and select the Related Population *Amastigotes*. Mark the option *Number of Amastigotes*.
 - iv. Name the Property Suffix *nAma* (Figure 32).
- b. Add the building block **Select Population**. This step will determine the population of infected cells.
 - i. Select the Population *Selected Cells 2*.
 - ii. Select the Method *Filter by Property*.
 - iii. Expand the options menu and select the parameter *Number of Amastigotes nAma*. Choose the mathematical signal *>* and type *0* in the correspondent field.
 - iv. Name the Output Population as *Infected Cells* (Figure 33).

Note: The distinction between infected and non-infected cells is based on the identification of amastigotes in each cell. Any cell with the number of amastigotes above zero is considered infected. Therefore, it is critical to have a good previous amastigote selection to avoid the inclusion of artifacts, which would cause an overestimation of the number of infected cells.

Note: The adequacy of the parameters used in the analysis can be accessed by applying them to an image of non-infected control wells since it should not identify amastigotes or infected cells.

8. To select the output results, add the building block **Define Results** (Figure 34).
 - a. Select the Method *List of Outputs*. Add the parameters calculated for each population filtered on the steps above.
 - b. To count cells, select the Population *Selected Cells 2*.
 - i. Mark the *Number of Objects* option.
 - ii. Select the *Individual Selection* at the *Apply to All* option.
 - iii. Additionally, calculate the Cell Area by selecting *Mean* in the corresponding option.
 - c. To count amastigotes, select the Population *Amastigotes*.
 - i. Mark the *Number of Objects* option.
 - ii. Select the *Individual Selection* at the *Apply to All* option.

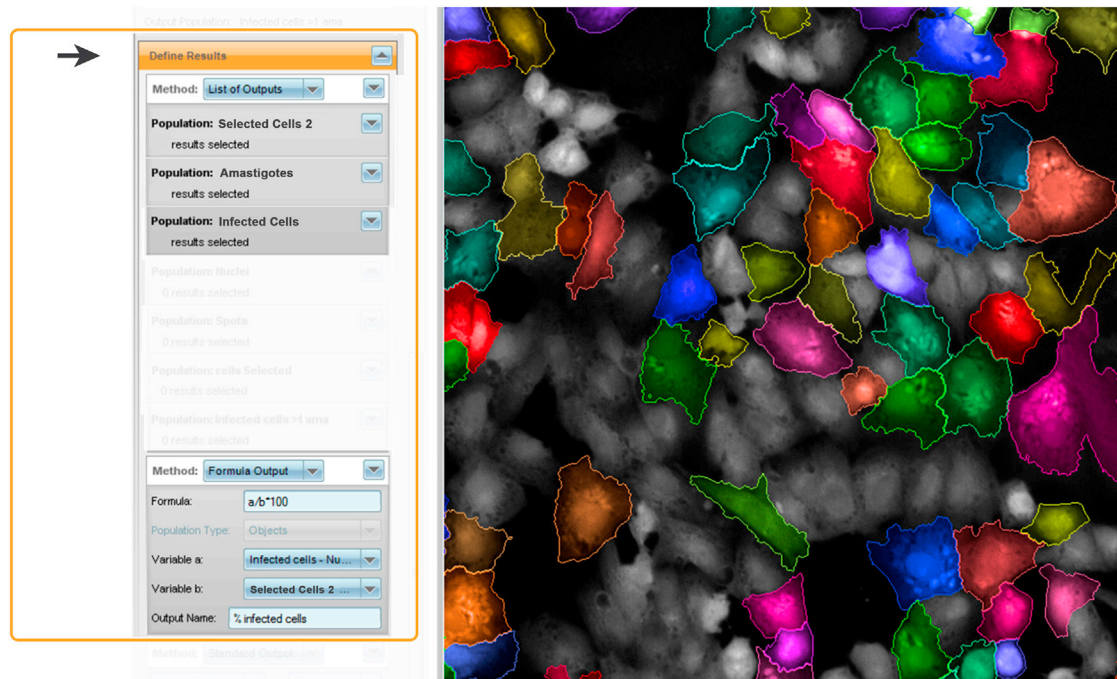


Figure 34. Screen visualization of parameters used to obtain a list of Results based on all defined properties

- d. To count infected cells, select the Population *Infected Cells*.
 - i. Mark the Number of Objects option.
 - ii. Select the Individual Selection at the Apply to All option.
 - iii. Additionally, determine the mean of amastigotes inside each cell by including the Number of Amastigotes and selecting to calculate Mean+StdDev. This mean will represent the results of the entire well on the output table.
- e. To calculate the ratio of infected cells, create a formula that will divide the total number of infected cells by the total number of cells from each well.
 - i. Select the Method Formula Output.
 - ii. Determine the "Variable a" selecting *Infected cells - Number of Objects*.
 - iii. Determine the "Variable b" selecting *Selected Cells 2 - Number of Objects*.
 - iv. Type the Formula: $a/b*100$.
 - v. Name the Output as *% infected cells*.

△ **CRITICAL:** Once the parameters for image analysis are defined, use them for all acquired images.

Data analysis from cell quantification

Once number of total cells, infected cells and total amastigotes is determined as demonstrated above, these data are analyzed to obtain the efficiency of the compounds that are being tested.

9. Calculate the Z-factor for plate validation.

This index provides information on the efficiency of infection and is used for validation of each plate of the assay, assuring that the replicas are similar enough and that non-infected (NI) and non-treated infected controls are different enough to provide reliable results. It is based on the relationship between the mean and the standard deviations (SD) of the ratio of infected cells

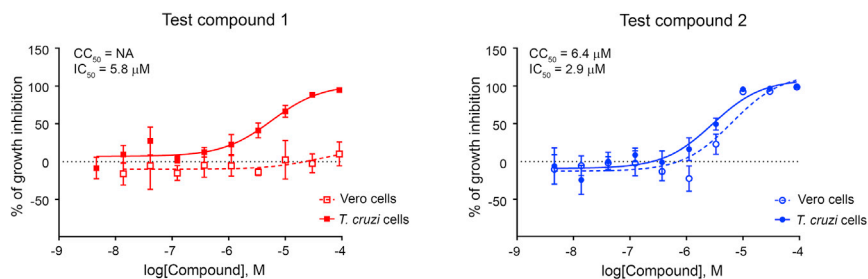


Figure 35. Dose-response curves obtained from 2 different compounds in Vero cells (CC_{50}) and in *T. cruzi* cells (IC_{50})
Data are represented as mean \pm SEM (standard error of mean).

in non-treated infected control wells (NT in Figure 18) vs. non-infected control wells (NI in Figure 18), as formulated by Zhang et al.¹⁰ This calculation is done automatically by the software and is depicted in the equation

$$Z = 1 - \frac{3SD \text{ of NT} + 3SD \text{ of NI}}{|\text{mean of NT} - \text{mean of NI}|}$$

where NT and NI values refers to the ratio of infected cells, calculated by dividing infected per non-infected cell populations in each well, transformed as percentages. A factor above 0.5 indicates an excellent assay, according to the authors' interpretation. See also troubleshooting section (problem 3 and problem 4).

10. Normalize data in each plate.

a) Data from total number of cells and amastigotes for each condition are normalized using untreated and non-infected wells as indicated:

- i. Normalized number of cells = $100 \times (1 - (\text{individual value}/\text{average of cells in NI and NT}))$.
- ii. Normalized number of amastigotes = $100 \times (1 - (\text{individual value}/\text{average of amastigotes in NT}))$.

b) For datasets including replicates, calculate the mean of technical replicates and the standard deviation (SD) of the experimental values. With these values, determine CC_{50} and IC_{50} for cells and parasites, respectively, by using non-linear regression in GraphPad Prism (Figure 35). The selectivity index is calculated by dividing CC_{50}/IC_{50} values.

Note: Even if other parameters such as percentage of infected cells or quantity of parasites per cell were not used in our analysis pipeline, they can be explored to compare control and treated cells. The quantity of parasites per cell, for example, can be used to corroborate if the tested compound acts mainly on parasites since the number should be smaller in treated cells than in untreated control cells.

Processing of images using the Living Image Software

Regions of interest (ROI) should be selected to the full body including tail. Background auto luminescence is calculated using the same ROI size, whose value is used to subtract from each animal ROI value (Figure 36). All calculations are done using total flux (photons/second) and image units are expressed as average radiance (photons/sec/cm/steradian).

Raw data is tabulated, and statistical analysis is performed by using one-way ANOVA with spherical data and Tukey's multiple tests in GraphPad.

LIMITATIONS

The described techniques are limited to the availability of specialized materials and equipment, like the high-content imaging system, live imaging system and even 8- and 12-channel micropipettes.

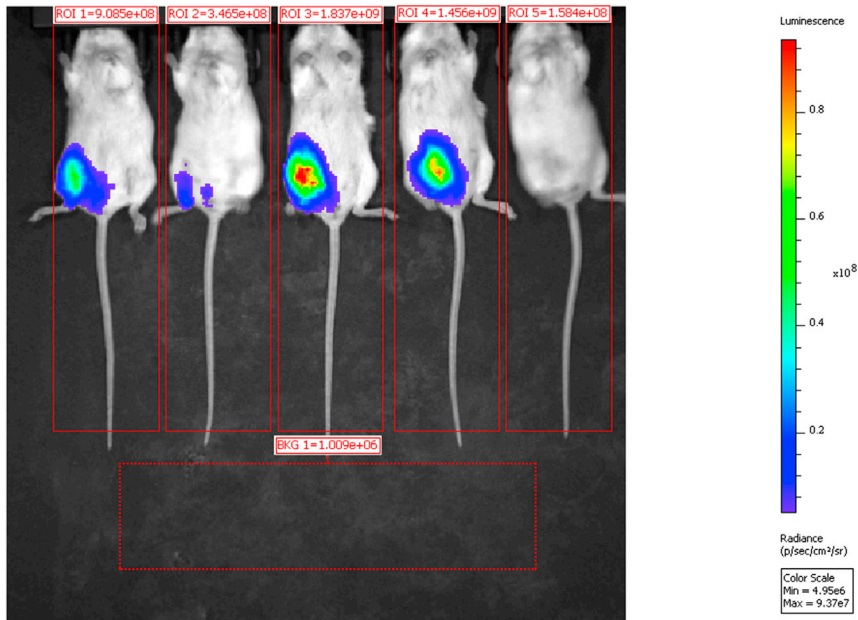


Figure 36. Example of ROI selection to include the entire animal body

The lower rectangle with the same size of the mice ROI is used to determine the background.

T. cruzi strains other than Dm28c may require previous testing to assess infection rates, time required for intracellular parasite release, *in vitro* MOI to reproducible and sufficient infection rate and parasite count to *in vivo* inoculation.

Even though *in cellulo* and *in vivo* infection models can together provide reliable information about the efficacy and toxicity of the tested compounds, they still remain study models. Some compounds selected as promising targets may present poor results in the following stages of clinical trials.

TROUBLESHOOTING

Problem 1

Vero cell culture has more than 15 passages (step 10 - [Obtaining Vero cells for infection experiments](#)).

Potential solution

- Thaw a new aliquot and restart cultivation

Problem 2

Releasing of cell-derived trypomastigotes do not reach 5×10^5 trypomastigotes/mL (steps 2 and 3 - [Obtaining a stock of infective trypomastigotes *T. cruzi*](#)).

Potential solution

- Check passage number of Vero cells. If more than 15 passages, thaw a new aliquot and restart cultivation.
- Check temperature and CO₂ conditions of the incubator.
- Check the pH of culture medium. Low pH may accelerate the differentiation of released trypomastigotes into non-infective extracellular amastigotes.

Problem 3

Z-factor value lower than 0.5.

Potential solution

- Check if cell artifacts are being spuriously included as spots in the amastigote selection. Address this by analyzing the parameters of amastigotes identification in the non-infected wells.
- Check the reproducibility of replicas.
- Guarantee that non infected control cells are indeed not infected by *T. cruzi* cells by avoiding cross-contamination between wells.
- Check passage number of Vero cells. If more than 15 passages, thaw a new aliquot and restart cultivation.
- Check if the infection ratio is adequate for the selected cell model and *T. cruzi* strain.

Problem 4

Low homogeneity between replicates at the *in cellulo* analysis.

Potential solution

- Be cautious and gentle when pipetting, avoiding touching the bottom of wells as indicated in [Figure 3](#) (all pipetting steps from Intracellular *T. cruzi* inhibition in infected Vero cells – first screening of the compounds and Intracellular *T. cruzi* inhibition in infected Vero cells – deep investigation). Adequacy of the pipetting procedures to avoid cell loss can be evaluated before starting by performing some rounds of washing tests using a 96-well plate with adhered Vero cells and check the cell abundance in washed versus non-washed wells on an inverted light microscope.
- Include more replicas in each assay (Intracellular *T. cruzi* inhibition in infected Vero cells – first screening of the compounds and Intracellular *T. cruzi* inhibition in infected Vero cells – deep investigation).
- The cell washing and pipetting steps could be done by the same person along the complete experiment.

Problem 5

The curve from the inhibition data does not have the characteristic S-shape.

Potential solution

- Verify if the problem is due to compound concentration too low or too high and use another starting concentration or include new dilution points.

Problem 6

The aspect of the parasite culture appears to have changed throughout time.

Potential solution

In our experience, transfected *T. cruzi* epimastigotes strains, are considered homogeneous and can be maintained for indefinite passages, provided that the culture medium is always supplemented with the corresponding selection antibiotic. As soon as a newly transfected strain is confirmed by molecular techniques, stocks must be prepared and stored for long-term in liquid nitrogen. If any abnormal behavior is detected (e.g., growth impairment, atypical phenotype, contamination), that culture should be terminated and a new one should be started from the stock and tested for the expression of the gene of interest.

Problem 7

The cell culture appears to be contaminated.

Potential solution

Potential contamination of the cultures must be constantly monitored by observing for changes in its physical properties (color, turbidity, particles), and by inspecting periodically (e.g., at least in each harvest) under a light microscope searching for the presence of yeast and bacteria. Prevention of contamination is achieved by keeping the highest best practices standards in manipulating all cultures, and all materials must be kept under strict aseptic conditions. Additionally, the culture medium should be supplemented with general purpose antibiotics. If, however, any contamination is detected, the culture must be discarded, and the assay restarted. All materials must be replaced or at least inspected and tested for contamination as well.

Problem 8

The mice infection is not detected at 3 dpi.

Potential solution

- Check the effectiveness of the bioluminescence assay by *in vitro* analysis. When adding luciferin to the parasites in PBS, luminescence should be readily detected using a luminescence detector. Be sure to use parasites without D-luciferin as a negative control. Check for quenching effects of anesthetics on bioluminescence.¹¹
- Check for expression of luciferase in your *T. cruzi* population by qPCR and Western blotting.
- Check for luciferin integrity by testing it in another luciferase assay.
- If all the above are correct, then check for other signals of *T. cruzi* infection in mice. Both conventional and molecular assays for indirect or direct *T. cruzi* detection can be used. Additionally, at 7 dpi the animals should start presenting altered behavior plus other clinical signals of *T. cruzi* infection.
- If the above assessments are correct, absence of infection signal at 3 dpi could be indicative of low infective potential of the inoculated parasites, both due to poor preparation or to long waiting times until inoculation.
- In either case, the assay should be discontinued, and another infection must be performed using another group of animals.

Problem 9

The luminescence intensity signal is higher in the treated than in the control group.

Potential solution

- The inoculated dose of the compound might be toxic to mice. Reduce dosage according to IC₅₀.
- Try other inoculation routes like oral gavage or subcutaneous. Be sure to obtain permission from the Animal Experimentation Ethics Committee for those changes prior to any operational alteration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nilson Ivo Tonin Zanchin (nilson.zanchin@fiocruz.br).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate dataset or original code. Any additional information required is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

E.P.G.-S. and G.F.A.P.-C performed the experiments. E.P.G.-S., G.F.A.P.-C, M.M., C.R., W.S., and N.I.T.Z. designed the experiments. E.P.G.-S., G.F.A.P.-C., and N.I.T.Z. analyzed data. E.P.G.-S., G.F.A.P.-C., and N.I.T.Z. wrote the paper. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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