Imaging of *Trypanosoma cruzi* infected 3T3 fibroblasts, developing a 384 well assay

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INTRODUCTION

Chagas disease (CD) is caused by the protozoan parasite, *Trypanosoma cruzi*. (Fig 1). CD is endemic to Latin America and has been recognized by WHO as one of the world's 13 most neglected tropical diseases[2]. Only two drugs are available to treat CD and both have associated toxicity with severe side effects, strain resistance and often treatment is unsuccessful. There is an urgent need for novel and less toxic compounds for the drug discovery pipeline. Existing assay technologies for screening compounds[3,4] against *T. cruzi* infective forms have limitations. We aim to improve upon existing assay formats by defining an infected host cell with fluorescent markers.

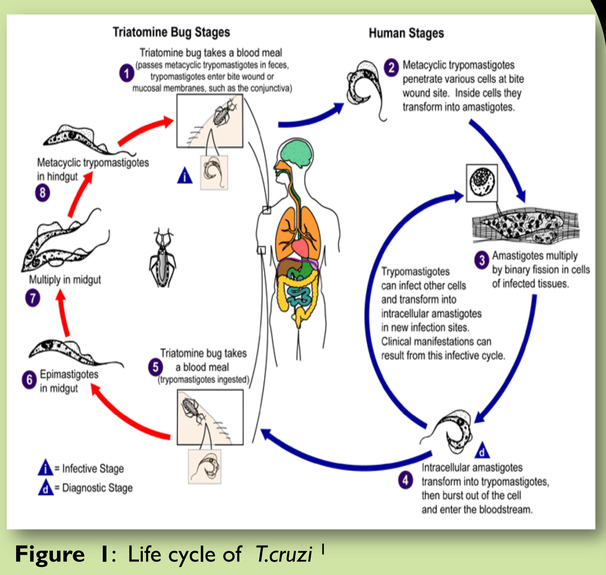

Figure 1: Life cycle of *T.cruzi* [1]

Ultimately, this assay format would include both the amastigote and trypomastigote forms of *T. cruzi*.

MATERIALS AND METHODS

Maintenance of Parasite Life Cycle

$4x10^5$ 3T3 cells were inoculated in to $25cm^2$ flasks, in phenol free RPMI+ 10% FCS . Following 24h incubation at 37°C and 5% CO_2, $4x10^6$ *T.cruzi* Tulahuen strain trypomastigotes were inoculated on to the cell bed. After 24 hr incubation, supernatant was removed and replaced with growth media. After a further 3-4 days, liberated trypomastigotes were harvested from the supernatant.

Co-culture of 3T3 Cells and Parasite in 384 Well Plates

$1x10^3$ cells were inoculated in to wells. Following 24h incubation, trypomastigotes were inoculated on to the cell bed. After 24 h incubation, supernatant was removed and replaced with growth media. After either a further 2 or 6 days, wells were processed for imaging.

Fluorescent Stains

Stains were added following either 3 or 7 days co- incubation, excepting CellTracker™ Green, which was incubated with trypomastigotes for 45 mins, at 5µM, before plate inoculation.

RESULTS

7 Day Co-incubation Assay Development

With an MOI of 10:1, the level of % infected cells and trypomastigote liberation suggested one cycle of infection may occur in a 7 day co-incubation of parasite and host cells (Fig 2).

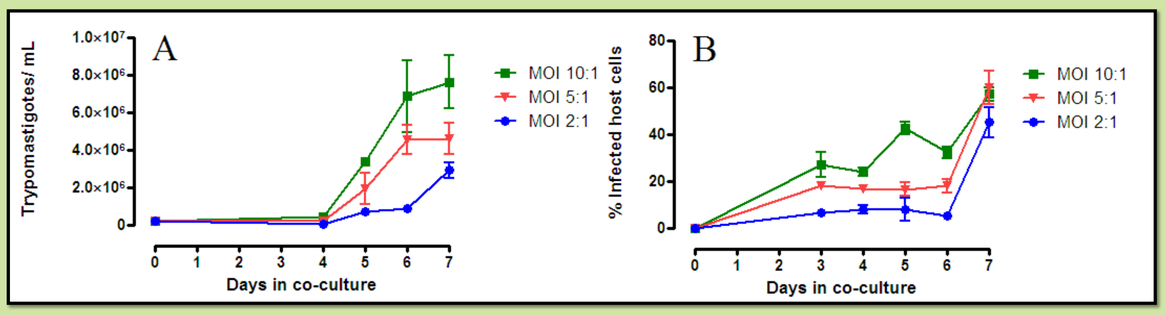

Figure 2. Production of (A) amastigote infected cells and (B) trypomastigotes in a 384 well plate following 7 days incubation of *T.cruzi* with 3T3 host cells. An decrease of total infected cells on day 6, coupled with an increase in trypomastigote prodution could suggest that this is the first release of trypomastigotes. An increase in infected cells day 6 suggests that these trypomastigotes may re-infect host cells.

Presence of external amastigotes made the definition of an infected cell difficult by imaging methods (Fig 3). Host cell viability was monitored over 7 days and did not decrease (results not shown) and cell bursting due to host cell death was not the cause.

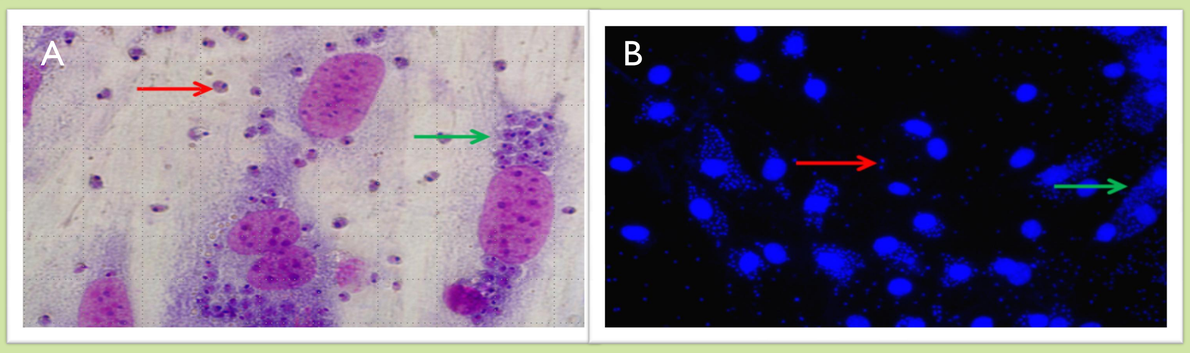

Figure 3. External amastigotes in a 384 well plate following 7 days co-incubation of *T.cruzi* and 3T3 host cells. (A) Geimsa stain in a 384 well plate at 160x magnification (B) Hoechst nuclear staining of fixed, infected 3T3 cells in a 384 well plate at 100x magnification. Green arrow= internal amastigoes, red arrow=external amastigotes.

3 Day Co-incubation Assay Development (Amastigote forms only)

No interference was observed from external amastigotes following 3 days co-incubation of 3T3 cells and *T.cruzi* parasite at an MOI of 10:1. A range of dyes were assessed for the ability to define an amastigote infected 3T3 cell (Table 1).

Dye	Fixation	Host cell	Amastigotes
Hoechst+ HCS CellMask Red or HCS CellMask Blue	✓	×1	✓
Hoechst	×	✓	×
Hoechst/ NuclearMask/DAPI (+permeabilisation)	×	✓	×2
C_{12} -Resazurin	×	✓	×
Cell Tracker™ Green CMFDA	×	×	✓
Cell Tracker™ Green CMFDA +Hoechst + C_{12} -Resazurin	×	✓	✓

(1) Cells stain, but easily lift from well surface during processing. **(2)** 0.1% triton x-100, cells wash from plate, 0.1% poor resolution

Table 1: A range of nuclear and cytoplasmic markers used to stain infected host cells.

Infected Cells Image Analysis CellTracker™+ Hoechst + C_{12}-Resazurin

MBF Image J was used to overlay images taken from a 3 day incubation of *T.cruzi* and 3T3 cells (Fig 4: A1, B1, C1). Acapella ® was used to estimate the host cell borders (Fig 4: A2, B2 C2). Spot analysis was used to estimate parasites within the host cell borders and revealed the greatest number of spots was seen clustered in infected cells (A3) versus non-infected cells (B3) and puromycin treatment (C3).

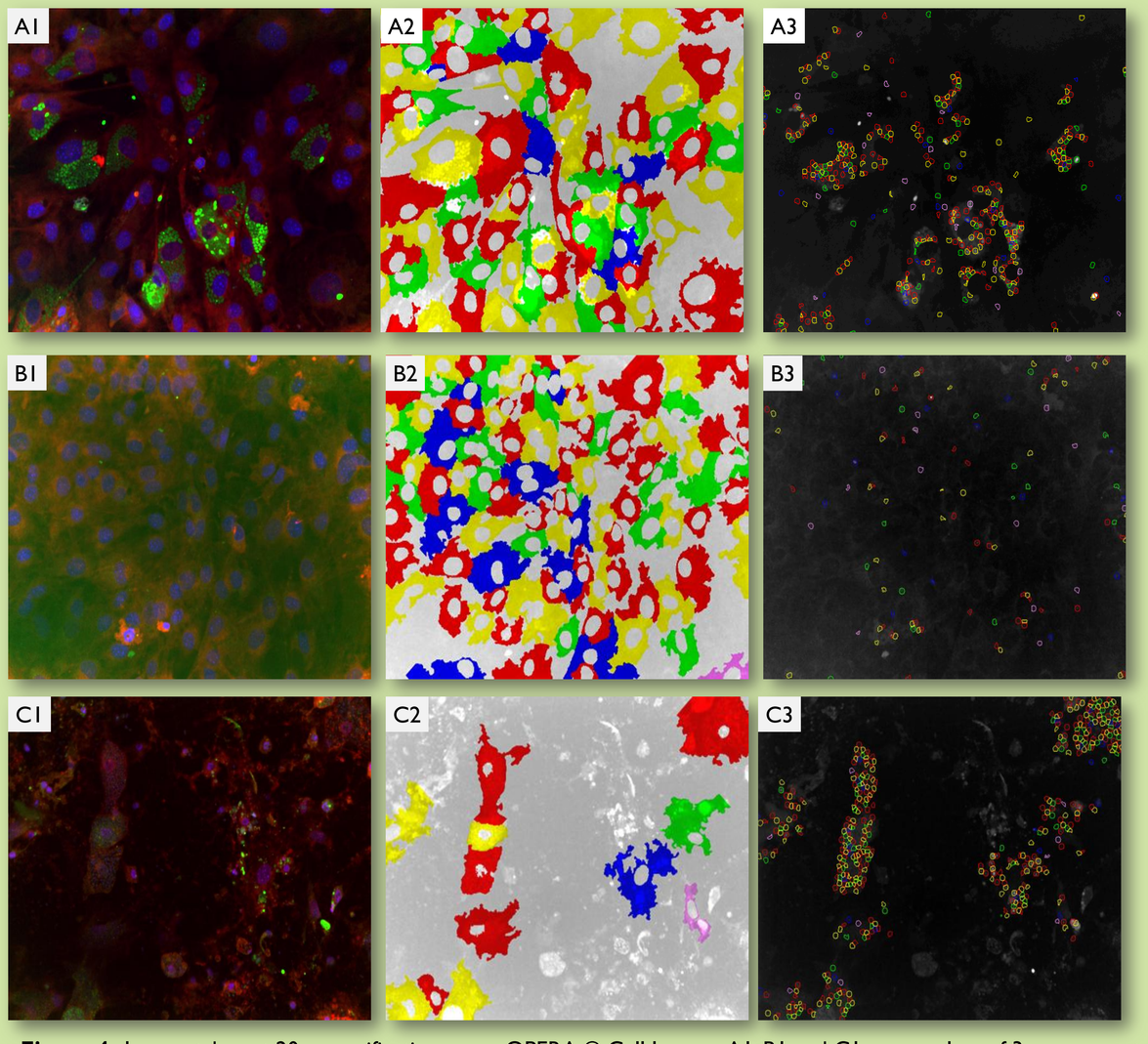

Figure 4: Images taken at 20x magnification on an OPERA ® Cell Imager. A1, B1 and C1 are overlays of 3 exposures to visualise 3T3 host cell infection, at Ex/Em 405/455=Hoechst to define host cell nuclei, designated blue; 532/ 600=C_{12}-Resazurin to define host cell cytoplasm and as an indicator of host cell viability, designated red; 488/520 CellTracker™ Green to define *T.cruzi* host cell internalised amastigotes, designated green. A= infected host cells, B= non-infected host cells and C= 24 hours exposure of infected host cells to 100nM puromycin. A2, B2 and C2 represent the cytoplasm of host cells defined by host nuclear and cytoplasm analysis using Acapella ®. A3, B3 and C3 represent the number of spots inside host cells, defined by Acapella® spot analysis.

DISCUSSION

It was not possible to incorporate an imaging based assay in to a 7 day co-culture of 3T3 and *T. cruzi* cells, due to the presence of external amastigote forms. Thus, an assay format using a 3 day co-culture of 3T3 and *T.cruzi* cells was successfully investigated.

A 3 dye, live cell system was able to effectively define an infected host cell.

Images were analysed with Acapella® demonstrating the presence of more infected cells in untreated wells in comparison to puromycin treated wells and non- infected cells, as desired.

Future optimisation includes improvements to the Acapella ® algorithm to include number of infected cells with >10 spots constituting an infected cell (current output is number of spots), along with the fluorescent intensity of host cells to estimate viability.

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