The Development of a Clinically Relevant, High-Throughput High-Content Imaging Assay For Leishmania

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Introduction:

Leishmaniasis is caused by protozoan parasites of the genus Leishmania. The disease is prevalent in over 90 countries and manifests as one of 3 forms: viseral, mucocutaneous or cutaneous. Viseral leishmaniasis is fatal without treatment while mucocutaneous and cutaneous are extremely disfiguring.

The parasite is transmitted by sandflies and following infection the metacyclic promastigotes are engulfed by macrophages where they transform and multiply as amastigotes causing the clinical symptoms of leishmaniasis.

The only drugs available are toxic with long administration schedules and resistance is a growing concern. A efficacious, non-toxic, easily administrable drug is urgently needed.

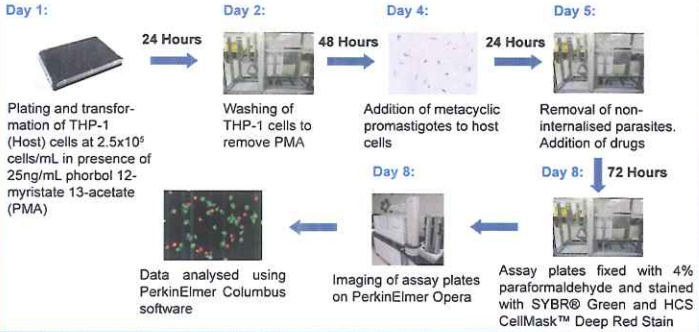
Numerous assays have been developed using a variety of life-cycle stages, assay platforms and technologies. However, these assays are often not clinically relevant, poorly reproducible and frequently *in-vitro* activity does not translate to *in-vivo* efficacy.

Aim:

To develop a high-content, high-throughput imaging assay to enable indentification of compounds against *Leishmania donovani* DD8 intracellular amastigotes.

Methods:

The leishmania intracellular amastigote assay invovles multiple steps over a 8 day period, summarized below.



Results:

1. Determination of Optimal Host Cells

The macrophage cell lines; Raw 264.7, J774.1 and THP-1 were extensively profiled in terms of growth rate, infectivity and amenability to high content image (HCI) analysis to determine the most suitable cell line for use in the assay.

Figure 1: Whole well montages (64 fields) of Raw 264.7 ($4x10^3$ cells/mL) (A), THP-1 ($2.5x10^5$ cells/mL) (B) and J774.1 ($4x10^3$ cells/mL) (C) cells seeded in 384 well CellCarrier-384 optically clear bottom tissue culture treated plates (PerkinElmer), stained with HCS CellMask™ Green, imaged on a PerkinElmer Opera at 20X magnification, exposure 1, camera 2 Em 540 nM. The non-contact inhibited growth and morphology of RAW 264.7 (A) and J774.1 (B) cells meant that the cell lines were not compatible with a HCI based assay. THP-1 cells (B) were therefore selected as the host cells in the assay.

2. Selection of Nuclear Acid Stain

Five commercially available nucleic acid stains were evaluated for their ability to selectively stain host cell and parasite nucleic material.

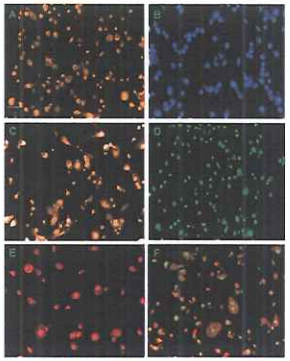


Figure 2: THP-1 cells, $2.5x10^5$ cells/mL infected with *L. donovani* DD8 metacyclic promastigotes at a multiplicity of infection of 1:5 (host cells to parasite), imaged on a PerkinElmer Opera at 20x magnification. Stained with: A) Acridine Orange (10nM), staining was non-specific B) Thiazole Orange (2.1uM), non-specific staining was observed. C) Draq 5 (5uM), specific staining of host cell and parasite nucleic material was observed but the signal to background was low. D) Hoechst (10uM), specific staining of host cell and parasite nuclei observed but difficult to detect parasites within host cell cytoplasm. E) SYBR® Green (1/10000), specific staining of host cell and parasite nuclei and a large signal to background coupled with a high flurorescent intensity at low concentrations led to this dye being selected. F) SYBR® Green and HCS CellMask™ Deep Red Stain overlay. SYBR® Green when used in combination with HCS CellMask Deep Red easily and clearly allowed the leishmania amastigotes to be visualised within the host cell nuclei.

3. Script Development

In order to identify lesihmania amastigotes within host cells and thus quantify the number of infected cells an image analysis script was developed using the building blocks within the PerkinElmer Opera Columbus software.

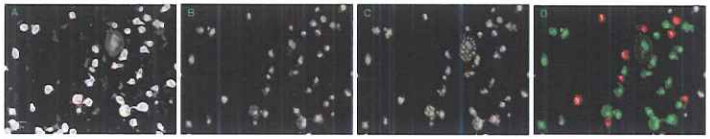
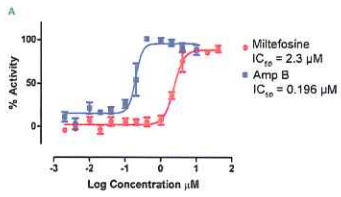


Figure 3: A)The find cytoplasm building block was used to identify the cytoplasm of the host cells using the HCS-CellMask™ Deep Red stain captured on exposure 1, camera 3, Em 690 nm B) Host cell nuclei were identified using the find nuclei building block using the SYBR® Green stain image captured on exposure 1, camera 2, Em 540 nm. Size exclusion was used to excluded non-vaible host cells. C) The find spot tool was utilised to identify amastigotes present within the cytoplasm of the THP-1 host cells using the SYBR® Green stain. D) Host cells with greater than 3 'spots' (amastigotes) in the cytoplasm were classed as infected (Highlighted in green).

4. Establishing Reference Drug Activity

A panel of reference compounds currently used for the treatment of leishmaniasis were screened using the newly established *L. donovani* DD8 intracellular amastigote assay.



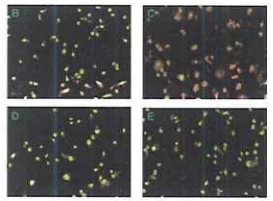


Figure 4: A) The dose-response curves of miltefosine and amphotericin B against intracellular *L.donovani* DD8 amastigotes. The IC_{50} values were comparable to previous reported values. THP- cells ($2.5x10^5$ cells/mL) infected with *L. donovani* DD8 amastigotes at a multiplicity of 1:5 stained with SYBR® Green and HCS CellMask™ Deep Red imaged on a PerkinElmer opera at 20X. Following treatment with 10µM of miltefosine (B) and amphotericin B (C) no amastigotes are detected within the cytoplasm of host cells. In contrast following treatment with 0.1µM of miltefosine (D) and amphotericin B (E) amastigotes could clearly be detected within the cytoplasm of host cells.

Acknowledgements:

The work was financially support by the Drugs for Neglected Diseases *Initiative* (DNDi). Dr A Jones received a Tony B Academic Award from The Society of Laboratory Automation and Screening (SLAS) to attend SLAS 2015.

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