Exploiting genetically modified dual-reporter strains to monitor experimental *Trypanosoma cruzi* infections and host:parasite interactions

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Abstract

Trypanosoma cruzi is the causative agent of Chagas disease, the most important parasitic infection in Latin America. Despite a global research effort, there have been no significant treatment advances for at least 40 years. Gaps in our knowledge of *T. cruzi* biology and pathogenesis have been major factors in limiting progress. In addition, the extremely low parasite burden during chronic infections has complicated the monitoring of both disease progression and drug efficacy, even in predictive animal models. To address these problems, we genetically modified *T. cruzi* to express a red-shifted luciferase that emits tissue-penetrating orange-red light. Mice infected with these highly bioluminescent parasites can be monitored by *in vivo* imaging, with exquisite sensitivity. This technology has allowed chronic infections to be followed in real-time, identified the gastro-intestinal tract as a site of long-term parasite persistence, revealed the infection dynamics of genetically modified parasites, and played a central role in Chagas disease drug development programmes. However, a major drawback of bioluminescence imaging is that it does not allow visualisation of host:parasite interactions at a cellular level. To facilitate this, we generated *T. cruzi* strains that express a chimeric protein that is both bioluminescent and fluorescent. Bioluminescence allows the tissue location of infection foci to be identified, and fluorescence can then be exploited to detect parasites in histological sections derived from excised tissue. In this article, we describe in detail the *in vivo* imaging and confocal microscopy protocols that we have developed for visualising *T. cruzi* parasites expressing these dual-reporter fusion proteins. The approaches make it feasible to locate individual parasites within chronically infected murine tissues, to assess their replicative status, to resolve the nature of host cells, and to characterise their immunological context.

Key words: *Trypanosoma cruzi*, chronic Chagas disease, murine models, *in vivo* imaging, confocal microscopy, bioluminescence, fluorescence

1. Introduction

In Latin America, between 5 and 10 million people are infected with the protozoan parasite *Trypanosoma cruzi*, the aetiological agent of Chagas disease **(*1, 2*)**. Throughout the region, it is a leading cause of heart disease. Because of migration, Chagas disease is also becoming a global public health concern, with for example, an estimated 300,000 infected individuals in the US **(*3*)**, and 100,000 in Europe **(*4*)**. In endemic regions, the parasite is spread predominantly by hematophagous triatomine bugs, although other means of transmission can include contaminated food and drink, organ transplantation, blood transfusion and the congenital route.

T. cruzi is an intracellular parasite with an extremely wide host range, and has an ability to infect most mammalian cell types. In humans, the infection, which is considered to be life-long, passes through three defined stages. The initial acute phase begins 2-4 weeks post-infection, with parasites becoming widely disseminated in tissues and organs. Although the symptoms are usually mild, and resolve within a short time period, there are occasional fatalities due to myocarditis or meningoencephalitis. In the majority of cases, a robust immune response mediated by $CD8^+$ IFN-γ^+ T cells controls the infection **(*5, 6*)**, which then proceeds to the asymptomatic chronic stage, where the parasite burden is extremely low and difficult to monitor. About 30% of those infected eventually develop debilitating chronic stage pathology, although this can take decades to become symptomatic. Most commonly, these individuals suffer from cardiomyopathy, and less frequently digestive tract megasyndromes **(*7-9*)**.

In those who develop chronic Chagas disease pathology, *T. cruzi* parasites are present in very small numbers and exist predominantly as intracellular amastigotes, with undefined tissue distribution. This scarcity of parasites poses the questions – what actually drives disease pathology, and why is there such a wide spectrum of outcomes? For many years, autoimmunity was widely considered to be an important mechanism. This hypothesis was based on observations that detectable *T. cruzi* are frequently absent in heart tissue derived from patients with cardiomyopathy, that host and parasite proteins can display cross-reactive epitopes, and that acute stage infections are characterised by polyclonal expansion of B- and T-cells, some of which may be autoreactive **(*10, 11*)**. However, there is now a community-wide consensus that the role of autoimmunity has been overstated and that the continued presence of the parasite is a pre-requisite for pathology **(*12, 13*)**. Other significant factors that have been associated with disease severity include host and parasite diversity, and immune status **(*14*)**. Identifying the determinants of pathology in chronic Chagas disease will have major implications for treatment strategies.

Progress in dissecting Chagas disease pathogenesis in humans has been hampered by the long-term nature of the infection, the complex and diverse outcomes, and by technical difficulties in establishing parasite burden and tissue distribution. Predictive animal models have therefore been of particular importance for research in this area. Recently, we developed a highly sensitive *in vivo* imaging system applicable to murine models of Chagas disease that exploits bioluminescent parasites that have been genetically modified to express an engineered red-shifted luciferase **(*15*)**. This system allows chronic infections to be monitored in real time for periods of more than one year. *Ex vivo* imaging revealed that acute stage infections were pan-tropic, with

all of the major organs and tissues highly infected. With progression to the chronic stage, the parasite burden fell by up to 3 orders of magnitude, with infections restricted predominantly to the stomach, colon and gut mesentery tissue, with other organs infected sporadically **(*16, 17*)**. Transient bioluminescent foci could also be detected in peripheral sites, including the skin, although intriguingly, these were highly dynamic, appearing and disappearing over a period of hours.

The production of luciferase-mediated bioluminescence by genetically modified parasites is an ATP-dependent process that requires the presence of the natural substrate luciferin. This mitigates against the use of this reporter in histological studies aimed at investigating host:parasite interactions at a cellular level. To overcome these limitations, we devised a “gene knock-in” strategy to generate parasites that expressed a dual-reporter fusion protein that was both bioluminescent and fluorescent **(*18*)**. For the fluorescence activity, we integrated a gene encoding the highly stable monomeric protein mNeonGreen in-frame with the *PpyRE9h* red-shifted luciferase sequence **(*19*)**. Using this dual-reporter system, the location of infection foci in infected mice can be determined by *in vivo* bioluminescence imaging, with fluorescence then being exploited to visualise individual parasites in histological sections derived from the excised bioluminescent tissue. For the first time, this has allowed us to routinely detect and image infected host cells in tissue samples derived from mice in the chronic stage of Chagas disease, when the parasite burden is extremely low. The approach is both flexible and widely applicable to experimental *T. cruzi* infections, and has the capability to provide new insights into parasite biology and the host immune response. Here, we describe detailed procedures that can be used to exploit the bioluminescence/fluorescence reporter system.

2. Materials

1. *T. cruzi* epimastigote growth medium: RPMI-1640 (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated (56^{o}C/30 minutes) fetal bovine serum (FBS) (BioSera), 0.5% (w/v) tryptone (Sigma-Aldrich), 0.5% (w/v) HEPES (Sigma-Aldrich), 0.03 M hemin (Biophoretics), 2 mM sodium glutamate, 2 mM sodium pyruvate, 2.5 units ml^{-1} penicillin and 2.5 μg ml^{-1} streptomycin (PenStrep, Gibco).

2. Hygromycin (InvivoGen).

3. L6 rat myoblast cell growth medium: *T. cruzi* epimastigote growth medium, without tryptone or hemin supplementation.

4. Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich)

5. ACK red cell lysis buffer (Gibco)

6. Euthatal (Merial)

7. d-luciferin (Syd Labs, Inc)

8. Isofluorane in oxygen (Merial)

9. Rely+On Virkon tablets (DuPont)

10. Absolute ethanol (analytical reagent grade).

11. Xylene Substitute (Sigma-Aldrich)

12. Paraffin wax – Histosec®60 pastilles (without DMSO) solidification point 58-60°C (Merck)

13. Tris buffered saline, pH 7.6

14. Nalgene Mr Frosty™ freezing container (Sigma-Aldrich)

15. Vectashield/DAPI (Vector Labs)

16. Histological cassettes and lids (Simport)

17. Microtome (Thermo Fisher Scientific)

18. ImmEdge hydrophobic barrier pen (Vector Labs)

19. *In situ* cell death detection kit, TMR red (Sigma-Aldrich)

20. 5’-Ethynyl-2’-deoxyuridine (EdU) (Thermo Fisher Scientific)

21. Click-iT Plus EdU Alexa Fluor 555 imaging kit (Thermo Fisher Scientific)

3. Methods

3.1 Parasite culturing

T. cruzi is highly infectious and all life-cycle stages, both *in vitro* and *in vivo*, are potentially hazardous (insect stage epimastigote cultures can contain infectious metacyclic trypomastigotes). The lower limit for an infectious dose should be regarded as one parasite. It is therefore essential that all procedures involving live parasites are performed in a microbiological safety cabinet (Class II) and that all "sharps" precautions are meticulously observed. The biological hazard classification of *T. cruzi* (both wild type and genetically modified strains) varies between countries. However, until parasites have been permanently inactivated, it is advisable to work under CL3 conditions, taking particular care when handling infected blood and tissue samples.

3.1.1. Culture epimastigotes of the bioluminescent/fluorescent *T*. *cruzi* CL-Luc:Neon strain in a closed cap culture flask at 28^{o}C in supplemented RPMI-1640 medium **(*20*)** (Materials), with 150 µg ml^{-1} hygromycin as the selective drug. Infectious metacyclic trypomastigotes (MTs) develop once the culture has entered stationary phase (approximately 2x10^{7} parasites ml^{-1}), and normally reach a frequency of ~10%.

3.1.2. Tissue culture trypomastigotes (TCTs) can be derived by infecting L6 rat myoblast cell monolayers (70% confluence) with stationary phase MTs, typically at a multiplicity of infection (MOI) between 1 and 5 (see note 1). Maintain the L6 cells in growth medium (Materials) in filter cap culture flasks at 37^{o}C. 18 hours post-infection, remove non-invasive parasites by washing the monolayer in plain

RPMI1640 pre-warmed to 37ºC, and then incubate with fresh L6 growth medium. Change the medium every 2-3 days until extracellular TCTs are abundant, usually 6 to 8 days post-infection. Harvest the extracellular TCTs from the medium by two-step centrifugation. First, spin at 100 *g* for 10 minutes in a 15 ml centrifuge tube, discard the pellet (mainly debris and detached cells), then centrifuge the supernatant at 1,600 *g* for 10 minutes to produce an enriched TCT pellet. Resuspend the pellet in 1 ml of L6 growth medium and count using a haemocytometer (use dilutions as necessary). Maintain the TCTs at 37ºC until required. To prepare infection inocula, centrifuge and resuspend the pellet in the required volume of DPBS.

3.1.3. To cryopreserve TCTs, adjust parasite density to $5x10^6$ ml^{-1} and add DMSO to 7.5%. Transfer aliquots to cyrovials and place them in a Mr Frosty™ freezing container. Store for 24 hours at -80ºC, then transfer to storage in liquid nitrogen. To revive parasites, transfer cryovials to a beaker containing tap water and thaw rapidly. Open the cap using a paper towel soaked in 70% ethanol. Transfer the contents to a 15 ml tube containing 9 ml of L6 growth medium and centrifuge at 1,600 *g* for 10 minutes to remove the cryopreservant. There should be a small pellet visible. Aspirate the supernatant taking care not to disrupt the pellet. Resuspend in 1 ml L6 growth medium, re-centrifuge at 10,000 *g* for 5 minutes, and resuspend the pellet in 250 μl medium. Incubate for 1-2 hours at 37ºC, then count using a haemocytometer (use dilutions as necessary). To prepare an infection inoculum, centrifuge and resuspend the pellet in the required volume of DPBS.

3.2 Infection protocols

All described procedures conform to the UK Animals (Scientific Procedures) Act 1986 (ASPA) and were originally performed under UK Home Office project licences (PPLs 70/6997 and 70/8207). The techniques outlined have been applied to BALB/c, C57BL/6 and C3H/HeN mice (purchased from Charles River, UK), and to severe combined immunodeficiency (SCID) CB17 mice (bred in-house). Animals are maintained under specific pathogen-free conditions in individually ventilated cages. They experience a 12 hour light/dark cycle and have access to food and water *ad libitum*. Routinely, we use female mice, aged 8-12 weeks at the point of infection. Male mice have been used in some experiments, without a noticeable difference in the infection profile **(*17*)**.

3.2.1. For standard experiments, we use $1x10^4$ *in vitro* derived TCTs (above) in 0.2 ml DPBS to infect SCID mice via i.p. inoculation. Load the parasites into a 1 ml syringe, attach a 25G needle and eject any air/bubbles. Pick up the mouse by the base of the tail with your dominant hand. Slowly and gently force the mouse down onto the surface of the cage top, while gathering up the skin with your thumb and index finger. When you have a secure grip, lift the mouse off the cage top and turn your hand over, exposing the belly. Restrain the tail with either the ring or the little finger. If performed properly, the mouse should unable to move its head too far from side to side. Wipe its abdomen with alcohol, then inject 0.2 ml i.p and return to the cage.

3.2.2. To monitor parasitemia, add 27 μl ACK red blood cell lysis buffer (Materials) into a 1.5 ml microfuge tube. Perform a tail snip or tail vein puncture, collect 3 μl

blood and mix with the ACK buffer using a P20 pipette. Wait 5 minutes, then count bloodstream form trypomastigotes (BTs) with a haemocytometer.

3.2.3. 16-19 days post-infection, when parasitemia has exceeded 10^6 ml^{-1}, collect blood from infected SCID mice by tail snip, tail vein puncture or cardiac puncture using a heparinized syringe. Check the parasite density by counting an aliquot as described above and adjust to $5x10^3$ BTs ml^{-1} in DPBS. Use the BT suspension directly for i.p. infection of immunocompetent mice. If necessary, prepare large volumes of inocula in a 50 ml centrifuge tube. Use a pastette to evenly distribute parasites in suspension between inoculations.

3.2.4. BALB/c mice are our main infection model and we typically infect with $1x10^3$ BTs via i.p injection. In other experiments, mice have been infected with up to $1x10^6$ MT, TCT or BT suspensions in 0.2 ml DPBS via i.p., i.v. or s.c. injection. The route of administration does not significantly affect the chronic infection profile **(*18*)**, although mice infected with low numbers of parasites ($<10^3$) take longer to reach the peak of the acute stage. Infections by the oral route, either by oral gavage of 0.2 ml into the stomach (intra-gastric route), or by deposition of 20 µl in the oral cavity using a pipette (buccal route), tend to be less efficient, although the subsequent infection profile is similar to that produced by injection **(*21*)**. Intra-gastric inoculation with BTs does not produce an infection. At experimental end-points, sacrifice mice by ex-sanguination under terminal anaesthesia (euthatal; 15 µl g^{-1} body weight).

3.3. *In vivo* imaging

3.3.1. For imaging, we use an IVIS Lumina II system (PerkinElmer) (as outlined in Figure 1A). Inject mice with 150 mg kg^{-1} d-luciferin i.p., leave for 5 minutes to allow luciferin to permeate all tissues, then anaesthetize using 2.5% (vol/vol) gaseous isofluorane in oxygen for 3-5 minutes.

3.3.2. Acquire images using LivingImage 4.5 software 10–20 minutes after d-luciferin administration (see notes 4-6). Exposure times can vary between 1 second and 5 minutes, depending on the intensity of the signal. Set binning to large, but reduce to medium or small if the signal reaches saturation (approx. 60,000 counts). Take dorsal and ventral images of each mouse (Figure 1B). Weigh the mice, revive in a cage or plastic box on a heated pad set to 37ºC, and then return to their cages. Under these imaging conditions, the limit of detection when parasites are inoculated i.p. is close to 100 parasites, with a linear relationship between parasite burden and whole body bioluminescence at 1000 parasites and above **(*16*)**. Our project licence allows each mouse to be imaged up to 40 times during its life-time.

3.3.3. To estimate parasite burden in live mice, draw regions of interest (ROIs) using LivingImage v.4.5 and quantify bioluminescence as total flux (photons/second) summed from dorsal and ventral images. Determine the detection threshold for imaging using control uninfected mice of similar weight and age.

3.4. *Ex vivo* imaging

3.4.1. As a preliminary, weigh the mice, image as above, allow them to recover, and return to their cages for a minimum of 1 hour. Note the location of bioluminescence foci and the likely tissue/organ involved.

3.4.2. When ready to perform necropsy and *ex vivo* imaging, inject with a second dose of d-luciferin (as above) and place the mouse in a spare cage. After 5 minutes, euthanize by injection of euthatal (as above). When unresponsive to pinching of the paws, pin the body onto a dissection board. Perform ex-sanguination by cardiac puncture, or alternatively cut the skin from abdomen to snout along the midline, then along the limbs, and exsanguinate by severing the axial vessels on the right side. Cut upwards from the midline of the peritoneum and thorax, and across both sides from the diaphragm to expose the heart and inner thorax region.

3.4.3. Prepare a solution of 0.3 mg ml^{-1} d-luciferin in DPBS, fill a 10 ml syringe and attach a 21G needle. Perfuse the heart via the left ventricle taking care not to remove the needle until perfusion is complete (see note 7). Clean up excess fluid/blood with pieces of absorbent paper towel and place in a discard bag.

3.4.4. Lay out the Petri dish and its lid, and using a pastette, transfer 1 ml of 0.3 mg ml^{-1} d-luciferin onto each, distributed evenly in 10-12 pre-allocated spots. Conduct the necropsy as outlined below and transfer each organ or tissue sample to the Petri dish according to the arrangement shown in Figure 2 (see note 8).

3.4.4.1. Remove the heart and dissect with a scalpel. Place each half into the dish in adjacent positions, exposing the internal structure, with the valves at the top. Remove the spleen and place in the dish with the thicker end to the left. Remove the lungs, a sample of the liver (from the largest lobe), a small sample of visceral fat (from the gonadal depot), and a sample of the skeletal muscle (left quadriceps).

3.4.4.2. Cut away the whole gut from the carcass, leaving the oesophagus attached to the stomach. Start by severing the oesophagus at the top of the thorax, then manoeuvre the scalpel into the abdomen by gently pulling from near the junction with the stomach. Detach the gut from the dorsal side of the peritoneum. Use two pairs of forceps to separate the mesenteries and detach them from the gut; as it is removed, simultaneously separate the oesophagus/stomach/small intestine from the caecum/colon. Position the colon in the dish as a straight horizontal line and position the connected caecum in a 'C' or 'S' shape, whichever is best to avoid kinks along its length. Transfer the stomach, oesophagus and small intestine as a single unit onto the dish. Position the distal end of the ileum first and gently guide the length of the small intestine into loops, positioned without gaps (as in Figure 2). Place the stomach slightly away from the ileum, with the oesophagus then arranged in a hook position, with all sections of the gut exposed as clearly as possible.

3.4.5. Use a pastette to add up to a further 1 ml of 0.3 mg ml^{-1} d-luciferin carefully over the organs/tissues, ensuring that they remain bathed in the solution, but without moving out of position. Transfer the Petri dish to the IVIS Lumina II, and image for 5 minutes on large binning (Figure 2).

3.4.6. When imaging is complete, transfer the Petri dish/lid back to a safety cabinet and prepare the samples for histology (see below). All samples should be washed in DPBS prior to transfer to tubes/histology cassettes. Depending on the experiment, collect a pre-determined set of samples or collect based on the bioluminescence location.

3.4.7. To estimate tissue parasite loads, use LivingImage software to draw individual ROIs around each organ and tissue sample. Include an ROI to represent a blank area of the dish to serve as a background value. Measure the bioluminescence intensity expressed as radiance (photons second^{-1} cm^{-2} sr^{-1}). Different tissues from uninfected control mice have slightly different background radiances, therefore normalize the data from infected mice using matching tissues from uninfected controls and use the fold-change in radiance (compared to the tissue-specific control reference values), as the final measure of *ex vivo* bioluminescence.

3.5. Carcass imaging

3.5.1. Following removal of the internal organs, position the carcass in the middle of a Petri dish lid, cut away and peel back the skin, exposing the internal cavity. Cut the skin along the thighs and legs and pull back to reveal as much of the muscle as possible. Finally, to expose the internal regions of the neck/head, cut the skin along the midline of the throat and diagonally up towards the ears, exposing the salivary glands, while peeling the skin back. Sever the ear canal and spread the skin flat against the Petri dish. Dissect the peritoneum from both sides and lay to the right of the carcass. Transfer the remaining liver, visceral and subcutaneous fat samples into the Petri dish lid alongside the carcass as shown in Figure 3A.

3.5.2. Using a pastette, carefully bathe the carcass/tissues in up to 1 ml additional 0.3 mg ml^{-1} d-luciferin solution. Transfer the Petri dish lid to the imaging chamber and image for 5 minutes on large binning. Bioluminescent emissions are remarkably

stable, and should remain readily detectable for more than 1 hour (Figure 3B), with d-luciferin solution being added to prevent drying out.

3.6. Preparation of tissue for histological analysis and examination by confocal microscopy

To ensure preservation of fluorescence in tissue samples derived from mice infected with parasites expressing the Luc:mNeonGreen fusion protein, we adapted methodology previously described by Nakagawa *et al.* **(*22*)**.

3.6.1. Identify fluorescent foci by *ex vivo* imaging (Figure 4A), and excise from tissue using a clean scalpel. Wash thoroughly with DPBS. For colon sections, place on filter paper and gently remove faecal pellets without tearing or damaging the tissue, using the angled seeker as a scraper. Fix tissue sections in pre-chilled 95% ethanol for 20-24 hours at 4^{o}C in histology cassettes.

3.6.2. Dehydrate the tissue at room temperature using four changes of 100% ethanol over a period of 1 hour, in a dark container placed on a shaker. To clear the sample, wash with two changes of xylene for 12 minutes each, continuing on the shaker in the dark container. Embed at 56^{o}C by placing the cassettes in two pots of melted paraffin for 12 minutes each. Once cooled, tissue/organ samples should be removed from the cassettes, gently positioned in wax block moulds and topped up with melted paraffin to prepare blocks for microtomy. Cool the wax blocks on a cold plate and store at room temperature in the dark to preserve fluorescence.

3.6.3. Cut a series of 3 to 10 µm thick tissue sections from each wax mould using a microtome and transfer the ribbon of sliced sections to a water bath. Select the desired section to mount and separate from the rest of the ribbon using tweezers, before easing the section onto a clean slide. Slowly dip the slide into a warm water bath (42-46°C), to separate the section from the slide, and reposition the section on the slide in the correct orientation using tweezers. Leave mounted slides flat to dry and then place in a metal rack overnight at room temperature. Place the metal rack on a hot plate (approximately 60^{o}C) for 30-60 minutes to melt away excess paraffin, and then store at room temperature. For confocal imaging, further de-paraffinize the slides with two changes (30 seconds each) of xylene, three changes (1 minute each) of pre-chilled 95% ethanol, and three changes (1 minute each) of pre-chilled Tris-buffered saline, pH 7.6 (TBS). Once dry, mount the slides using vectashield/DAPI, by pipetting an adequate volume on top of the tissue section. Carefully place a cover slip on top. Avoid bubbles and seepage of mounting fluid from the edges. Seal the edges using clear nail varnish and leave to dry, before storing at 4^{o}C in the dark until required.

3.6.4. For imaging (Figure 4B), we use a Zeiss LSM510 Axioplan confocal laser scanning microscope, with the associated software. First, scan the sections using the GFP/FITC filter to identify putative parasites. Check for the presence of kinetoplast (k) DNA (mitochondrial DNA) using the DAPI filter to distinguish parasites from any auto-fluorescent particles. Then, obtain phase images at 40x magnification to enable orientation of the tissue section and identification of specific layers/structures. Take images for DAPI and mNeonGreen and then switch to 63x or 100x objective and zoom-in on the parasites. Image in three dimensions (z-stacking)

to allow precise counting of amastigotes, with the appropriate scan zoom selected for the particular cell/number of parasites. Images can be analysed and scale bars added using the Zeiss LSM image browser software, and exported as JPEG or TIF files for analysis using other software such as image J (Figure 4B).

3.7 Assessing parasite replication: TUNEL assays

The TUNEL assay uses the enzyme terminal deoxynucleotidyl transferase to add fluorescently labelled UTP analogues to 3' hydroxyl groups generated at sites of DNA breaks **(*23*)**. It is routinely used to characterise apoptosis in mammalian cells. In trypanosomatids, this assay can be used to identify parasites undergoing kDNA replication at the time of tissue fixation **(*18, 24*)**. The kDNA consists of minicircles (~0.5-2 kb) and maxicircles (20-40 kb). The minicircles are present in thousands of copies per cell in a large catenated network **(*25*)**, reminiscent of chain mail armour. To be replicated, each circle must be detached from this network, and copied to produce two daughter circles, then re-attached. During this process, new strands transiently possess single strand DNA breaks. These nicks can be labelled using the TUNEL assay; therefore TUNEL positive kinetoplasts identify parasites actively replicating their mitochondrial DNA at the point of tissue fixation.

3.7.1. Deparaffinise slides as outlined in section 3.6.3. Draw around the relevant section with a hydrophobic pen, then permeabilize in 0.1% TritonX-100/PBS for 5 minutes. Wash 3 times with PBS. To each section, add an appropriate volume of TUNEL reaction mixture (10-50 µl for an average section; *In Situ* Cell Death Detection Kit, Materials) and overlay the slide with a coverslip to ensure that the reaction mix is evenly distributed. This also minimises the volume of reaction mix

required. Incubate the reaction for 1-2 hours at 37°C in a darkened humid chamber. Remove the coverslip by floating it off in PBS (this is easily done in a Petri dish), being careful not to damage the tissue section. Wash the slides three times in PBS and mount in Vectashield/DAPI. Examine the slides by confocal microscopy.

3.8. Assessing parasite replication: Nucleoside analogue incorporation.

Nuclear DNA replication can be measured by incorporation of nucleoside analogues *in vivo*, which are then detectable after fixation and sectioning **(*26-28*)**. Two methods are commonly used; incorporation of 5-bromo-2'-deoxyuridine (BrdU) followed by antibody detection, or incorporation of 5-ethynyl-2'deoxyuridine (EdU), which is detected by direct fluorescent labelling using copper-catalysed click-chemistry. The BrdU method requires harsh denaturation conditions to facilitate access of the antibody to the modified base. This is incompatible with maintaining the fluorescence of the mNeonGreen reporter protein. For this reason, we use EdU incorporation, since labelling can occur without DNA strand separation, and the click reaction has no effect on the fluorescent protein.

3.8.1. Administer EdU (12.5 mg kg^{-1} in DPBS) to infected mice by i.p. injection. To label replicating parasites, we inoculate EdU 24, 48 or 72 hours prior to sacrifice. To detect parasites which have undergone one round of replication, but then become dormant, we administer a single dose of EdU at least 7 days before.

3.8.2. After sacrifice, prepare tissue sections as described in sections 3.4. and 3.6 (Figure 5A). Deparaffinise slides as outlined in section 3.6.3, draw around the section with a hydrophobic pen, permeabilize in 0.1% TritonX-100/PBS for 5 minutes

and then wash 3 times with PBS. Prepare the Click-iT reaction cocktail (Materials) – as these kits are expensive, we prepare the minimum volume required for the sections being labelled. Only prepare the cocktail when the slides are ready for labelling, as it has to be added within 15 minutes. Add an appropriate volume of the reaction cocktail (up to 20 µl for an average section). Overlay the slide with a coverslip to ensure that the reaction mix is evenly distributed across the section. Incubate at room temperature for 30 minutes. Remove the coverslip by floating it off in PBS (in a petri dish), being careful not to damage the tissue section. Wash the slides three times in PBS and mount in Vectashield/DAPI. Examine the slides by confocal microscopy (Figure 5B).

Notes

1. To maximise the yield and purity of TCTs, use freshly seeded L6 cell monolayers and a MOI of 1. For infections on the same day, seed cells at $1.8x10^5$ ml^{-1}, incubate for 6 hours, then add parasites. For infections the following day, seed at $8x10^4$ ml^{-1} and incubate overnight.

2. To ensure reproducibility, we strongly recommended generating a large batch of TCTs that can be cryopreserved for future experiments.

3. Needles narrower than 25G may damage trypomastigotes and perturb infectivity. Avoid drawing parasites repeatedly through needles. To minimise inoculum leakage, employ proper injection technique. Place your gloved thumb over the injection site for 10-15 seconds after withdrawing the needle, keeping the animal angled with its head around 45 degrees downwards. To avoid the risk of self-infection, never place a syringe needle back into the sheath, instead put directly into a sharps bin.

4. Non-infected control mice should be imaged as part of each imaging session. These data allow establishment and monitoring of the background luminescence and also control for any effects of luciferase injections or anaesthesia on downstream read-outs, such as pathology.

5. The optimum imaging window is 10–20 minutes after injection of d-luciferin. However, the bioluminescence signal from live animals decreases relatively slowly and is still approximately 80% of the maximum after 30 minutes **(*15*)**.

6. For unbiased image analysis in LivingImage, adjust the pseudocolour scale levels to hide the bioluminescence signal before drawing ROIs.

7. For effective perfusion, ensure that the solution is at ambient temperature. Check that the needle is correctly positioned by visualising inflation of the heart chambers and then increase the pressure on the syringe very gradually, administering around 1 ml every 3 seconds. The lungs and liver should become paler. If this does not occur, re-position the needle, without removing.

8. Organs and tissue samples can be weighed prior to transfer into a Petri dish.

9. For a good quality confocal microscopy images, apply the Vectashield mounting solution to samples recently rehydrated with PBS. Apply 8–10 µl (depending on the size of the sample) in a continuous strip along the centre, and then lower the cover slip slowly from one end, without forming bubbles. Remove the excess mounting solution on the sides of the coverslip and seal with nail varnish.

10. For thin sections (<10µM), the TUNEL assay may label the nuclei of many mammalian cells **(*29*)**. This is an artefact due to the mechanical cleavage of DNA during sectioning, as mammalian cell nuclei may have a diameter that exceeds the thickness of the section. Amastigotes are too small for this to be a major problem, unless one is cleaved during the microtome sectioning. Labelling of replicating amastigotes should be restricted to the kDNA. Nuclear DNA labelling only occurs in dead or dying parasites after treatment with some drugs, depending on whether the mode of death induces DNA breaks in the nuclear genome.

11. EdU will also label replicating mammalian cells (Figure 5B), a phenomenon that is most obvious in sections of gut tissue, where epithelial cells are constantly replaced. This provides a useful positive control for labelling. In terminally differentiated tissue, such as muscle, there should be very few labelled host nuclei, an exception being infiltrating immune cells. EdU incorporation occurs during DNA synthesis and both kDNA and nuclear DNA will be labelled in replicating parasites, although the relative extent of this in each organelle will depend on the cell-cycle phase during EdU exposure. Labelled parasites have been detected in all tissue types so far examined, suggesting that EdU is widely distributed in mice.

12. For slides to be stained with antibody after labelling by TUNEL or EdU, this should be done after the TUNEL/EdU labelling reactions, prior to mounting the slides. These slides must be kept in the dark during all stages of antibody staining, as they already carry two fluorescent labels (mNeonGreen and TUNEL/EdU).

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Figure 1. *In vivo* imaging of *Trypanosoma cruzi* infection. (A) Outline of standard procedures. Mice are infected with bioluminescent parasites **(*16*)**, inoculated with luciferin at various time-points thereafter, with images acquired using the IVIS Lumina II system (PerkinElmer). Mice can be imaged multiple times over periods of longer than 1 year. (B) Using bioluminescence imaging to assess drug-treatment of chronic stage *T. cruzi* infection. BALB/c mice (day 14 post-infection) were treated with benznidazole (100 mg kg^{-1}, orally, once daily) for 5 days. They were monitored regularly by bioluminescence imaging (left hand image, non-treated mice; right hand image, benznidazole-treated). All mice were immunosuppressed on days 135, 139 and 143 using cyclophosphamide (200 mg kg^{-1} i.p). Heat-maps are on log10 scales and indicate intensity of bioluminescence from low (blue) to high (red).

Figure 2. *Ex vivo* imaging of tissues and organs from *Trypanosoma cruzi* infected mice, harvested at various points post-infection. In the chronic stage (day 112), parasites are restricted predominantly to the colon and/or stomach, with other organs/tissues infected only sporadically (see ref. ***17*** for further examples). Treatment with the immunosuppressive drug cyclophosphamide promotes expansion and dissemination of the infection. During the acute stage (day 14), all organs and tissues are infected. The inset (right) shows the arrangement of organs and tissues.

Figure 3. Imaging the carcass of mice chronically infected with *Trypanosoma cruzi*. (A) The carcasses from mice that were uninfected (left), infected (centre), and infected then immunosuppressed (left), were prepared for bioluminescence imaging as described in section 3.5. (B) Bioluminescence is remarkably stable over time.

Skin tissue containing bioluminescent *T. cruzi* foci were imaged over the time period indicated (minutes). Arrows show points when fresh d-luciferin was added.

Figure 4. (A) Bioluminescence imaging of organs from a chronically infected BALB/c mouse (day 117 post-infection). Organs were arranged as shown in Figure 2. A tissue sample (outlined in red) was excised from the stomach antrum and prepared for confocal microscopy (section 3.6.). (B) Images of tissue section obtained using the Zeiss LSM 510 confocal microscope. Upper images: low magnification showing the phase image for tissue structure, DAPI stained cell nuclei, mNeonGreen expressing parasites, and a merger of all three. The bar represents 50 µm. Lower images: Higher magnification of the area outlined in the dotted blue box showing mNeonGreen and DAPI merged images. The arrows indicate the kDNA (K), which appears as a rod-shaped DNA-containing structure in 2-dimensional images, indicative that the green fluorescent signals are derived from parasites. The image bars represent 5 µm.

Figure 5. (A) Bioluminescence imaging of organs from an acutely infected CB17 SCID mouse (day 15 post-infection) which was injected with 12.5 mg kg^{-1} EdU, 6 hours prior to sacrifice. Rectal tissue (circled in red) was excised and prepared for analysis by confocal microscopy. (B) Low magnification showing general DNA staining with DAPI, replicating DNA stained with EdU, parasites expressing mNeonGreen (the box indicates the cluster shown in C), and a phase image of the tissue. The final image was generated by merging all four channels. The bar represents 50 µm. In the EdU panel, the white arrows indicate replicating murine epithelial cells. Compare the epithelial layers to the terminally differentiated muscle

layer - EdU staining is specific to epithelial nuclei and absent from muscle cell nuclei. The white triangles indicate labelled *T. cruzi* clusters within the muscular layer, containing parasites that were undergoing DNA replication during the 6 hours following EdU administration. (C) Images of one of the parasite clusters at a higher magnification. The white arrows indicate that both the nuclear (N) and kinetoplast (K) genomes are labelled by EdU. The bar represents 10 μm.