A new chimeric triple reporter fusion protein as a tool for in vitro and in vivo multimodal imaging to monitor the development of African trypanosomes and Leishmania parasites

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14 **Short title**: A triple reporter for multimodal imaging of trypanosomatids

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16 Number of figures: 8 (+3)

17 **Highlights**

18 • New triple reporter combining a red luciferase and a tdTomato fused by a Ty1-tag

19 • Tested in two different stages of both *T. brucei* and *L. major* parasites

20 • Tested *in vitro* and *in vivo* in tsetse flies and murine models

21 • Useful for multimodal imaging at different scales, including intravital monitoring

22

Abstract

Trypanosomiases and leishmaniases, caused by a group of related protist parasites, are Neglected Tropical Diseases currently threatening more than 500 million people worldwide. Reporter proteins have revolutionised the research on infectious diseases and have opened up new advances in the understanding of trypanosomatid-borne diseases in terms of both biology, pathogenesis and drug development. Here, we describe the generation and some applications of a new chimeric triple reporter fusion protein combining the red-shifted firefly luciferase PpyREH9 and the tdTomato red fluorescent protein, fused by the TY1 tag. Expressed in both *Trypanosoma brucei brucei* and *Leishmania major* transgenic parasites, this construct was successfully assessed on different state-of-the-art imaging technologies, at different scales ranging from whole organism to cellular level, both *in vitro* and *in vivo* in murine models. For *T. b. brucei*, the usefulness of this triple marker to monitor the entire parasite cycle in both tsetse flies and mice was further demonstrated. This stable reporter allows to qualitatively and quantitatively scrutinize in real-time several crucial aspects of the parasite's development, including the development of African trypanosomes in the dermis of the mammalian host. We briefly discuss developments in bio-imaging technologies and highlight how we could improve our understanding of parasitism by combining the genetic engineering of parasites to the one of the hosting organisms in which they complete their developmental program.

1. Introduction

Vector-borne diseases affect about half of the World's population and are endemic in more than 100 countries. Among these vector-borne diseases, trypanosomiases and leishmaniases are considered as important Neglected Tropical Diseases (NTDs) that especially impact public health, accounting for ~24 million persons infected worldwide among over 500 million people at risk. These diseases are (i) Human African Trypanosomiasis (HAT) or sleeping sickness that is caused by the African trypanosomes *Trypanosoma brucei* (Buscher et al., 2017; Franco et al., 2017), (ii) Chagas disease or American Trypanosomiasis that is due to the American trypanosome *Trypanosoma cruzi* (Perez-Molina and Molina, 2017; Stanaway and Roth, 2015), and (iii) leishmaniases, a group of visceral and cutaneous diseases caused by a wide range of *Leishmania spp.* (Alvar et al., 2012; Matlashewski et al., 2014; Reithinger et al., 2007). In addition, some other trypanosome species (mainly *T. evansi*, *T equiperdum*, *T. congolense* and *T. vivax*) cause similar diseases in domestic animals and are responsible for important economic losses in developing countries (Rotureau and Van Den Abbeele, 2013). There is no vaccine against these parasites, most of the few currently available medications are not easily administrable and frequently present severe side effects, and drug resistance is dramatically increasing. However, in addition to mass screening and treatment programs, strategies for reservoir and / or vector control have proven their efficiency in certain endemic areas (Aksoy et al., 2017; Dias, 2015; Gonzalez et al., 2015).

In vivo intravital imaging methods have tremendously been improved over the last decades, and now represent powerful tools for the understanding of a large panel of dynamic biological processes, especially in the field of infectious diseases (Coombes and Robey, 2010). Beyond their obvious contribution to the ethical improvement of the experimental design of research studies on living animals, especially in terms of reduction and refinement, these approaches also offer a powerful and almost unbiased mean to monitor and compare the variable kinetics of infection among individuals. In the context of diagnostic tool and drug development, as well as in order to study the complex developmental cycles of trypanosomatid parasites, genetically engineered parasites have been developed to express a large panel of molecular reporters including fluorescent and bioluminescent probes both in *T. cruzi* (Lewis et al., 2015) and *T. brucei* (Burrell-Saward et al., 2015; Coles et al., 2017; MacLean et al., 2013; McLatchie et al., 2013; Van Reet et al., 2014), and especially in *Leishmania spp*. (Beattie and Kaye, 2011; Calvo-Alvarez et al., 2015a; Carneiro et al., 2017; Forestier, 2013; Lang et al., 2009; Taheri et al., 2015).

The choice of a given reporter is conditioned by that of a specific readout methodology that usually focuses on parasite detection at a single scale, *i.e.* at the cellular level in multi-well plates for drug screening, at the tissue level in *ex vivo* organ explants or models, at the entire

organism level to study the kinetics of the infection in lab animal models. However, only few attempts to combine these modes of detection and / or imaging have been proposed, especially in trypanosomatid research (Taheri et al., 2015). From the literature, dual reporter fusion proteins have been designed for the purpose of combining bioluminescence and fluorescence-based approaches: firefly luciferase Luc and EGFP (Patel et al., 2010; Taheri et al., 2015), firefly luciferase Luc2 and EGFP (Liu et al., 2010), firefly luciferase Luc2 and tdTomato (Liu et al., 2010), firefly luciferase Luc2 and TurboFP635 (Mezzanotte et al., 2014), mVenus and *Renilla* luciferase RLuc8, mTurquoise2 and RLuc8, mKusabiraOrange2 and RLuc8 (Takai et al., 2015). All these constructs encode for at least one marker emitting in the green. This is not optimal for intravital imaging due to the high absorption of the light at these wavelengths by the melanin and haemoglobin proteins present in tissues. To bypass this technical bias, McLatchie and colleagues have first reported a system that allows the detection of fewer than 100 bioluminescent *T. brucei* parasites in a murine model by the use of the codon-optimised red-shifted *Photinus pyralis* luciferase (PpyRE9H) (McLatchie et al., 2013). They were able to monitor in real time the parasite dissemination and to detect brain infections with a level of sensitivity *in vivo* that was significantly greater than achievable with a yellow firefly luciferase reporter (McLatchie et al., 2013). Almost at the same time, Van Reet *et al.* have compared the usefulness of three different red-shifted luciferases, and characterized their *in vitro* activity and growth profile, as well as their potential for *in vivo* murine infection follow-up in a set of diverse *T. brucei* strains (Van Reet et al., 2014). Burrell-Saward and co-workers also used the red bioluminescent *T. b. brucei* strain generated by McLatchie *et al.* to evaluate the efficacy of novel drugs *in vivo* against late-stage HAT and were able to detect drug relapse earlier than traditional methods (Burrell-Saward et al., 2015). In order to go a step further, we have selected and combined two reporters producing light in the red wavelengths (tdTomato at 581nm and red-shifted firefly luciferase PpyREH9 at 617nm) with a reduced absorption in tissues, therefore resulting in an enhanced sensitivity of the monitoring.

Common protocols, such as for electron microscopy, histology or protein biochemistry, can require deleterious heat and / or chemical treatments of the samples that frequently result in a denaturation of these recombinant proteins preventing further fluorescent emission, enzymatic activities as well as immuno-detection. To circumvent these obstacles, the Ty1 epitope has become a popular choice in trypanosomes, but also in other parasites such as *Leishmania* and *Toxoplasma* (Bastin et al., 1996). The Ty-tag is a 10-amino-acid sequence from the immunologically well-characterized major structural protein of the Ty1 virus-like particle of *Saccharomyces cerevisiae* (Brookman et al., 1995). The epitope is recognized by two different monoclonal antibodies, BB2 (IgG1) and TYG5 (IgA), that were tested in a variety of immunological conditions and that do not cross-react with trypanosome proteins.

116 Although a number of commercial antibodies from various companies are available against
117 either firefly luciferase or tdTomato, the Ty1-tag has been used extensively in
118 Trypanosomatids and it has already proved its usefulness in multiple contexts (Huet et al.,
119 2014; Julkowska and Bastin, 2009; Ooi et al., 2015). Therefore, between the two red-emitting
120 probes, we have inserted a small Ty1-tag (Bastin et al., 1996) that can be detected in most of
121 conditions, in order to obtain a stable cytosolic triple marker fusion protein
122 (PpyRE9H/TY1/tdTomato) that can be used to study the same sample by multi-modal
123 detection and imaging approaches.
124 Here, we describe the generation of this new chimeric triple reporter fusion protein. This
125 construct was successfully assessed on state-of-the-art imaging technologies both *in vitro*
126 and *in vivo* by using *Trypanosoma brucei* and *Leishmania major* transgenic parasites. This
127 approach allowed us to scrutinize the development of both parasites within the mammalian
128 host, as well as the journey of African trypanosomes in the tsetse fly digestive tract.

2. Material and methods

2.1 Leishmania major *and* Trypanosoma brucei brucei *strains and culture*

The AnTat 1.1E pleomorphic clone of *Trypanosoma brucei brucei* was derived from a strain originally isolated from a bushbuck in Uganda in 1966 (Le Ray et al., 1977). Procyclic forms (PCF) were grown at 27°C in SDM-79 medium (Brun and Schonenberger, 1979) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 8 mM glycerol (SDMG). Bloodstream forms (BSF) of the same strain were cultivated in complete HMI-11 medium (Kooy et al., 1989) at 37°C in 5% CO_2. For *in vitro* slender to stumpy BSF differentiation, we used 8-pCPT-2′-O-Me-5′-AMP, a nucleotide analog of 5'-AMP from BIOLOG Life Science Institute (Germany). Briefly, $2x10^6$ pleomorphic AnTat 1.1E slender forms were incubated with 8-pCPT-2′-O-Me-5′-AMP (5 µM) for 48 h (Barquilla et al., 2012). Freshly differentiated stumpy forms were then centrifuged and resuspended in SDMG medium with 10 mM glutathione for tsetse fly infection.

Leishmania major strain Friedlin V1 (MHOM/JL/80/Friedlin) (Kapler et al., 1990), was cultured at 26°C in M199 supplemented with 25 mM HEPES pH 7.2, 0.1 mM adenine, 0.0005% (w/v) hemin, 2 µg/ml biopterin, 0.0001% (w/v) biotin, 10% (v/v) FCS and antibiotic cocktail (50 U/ml penicillin, 50 µg/ml streptomycin).

2.2 Construction of the chimeric fusion DNA sequences

A 3.1 Kb sequence coding for the chimeric multiplex reporter protein PpyRE9H/TY1/tdTomato was genetically engineered. The resulting cytoplasmic reporter is composed of three distinct markers: the red-shifted luciferase (PpyRE9H) is fused to the tdTomato red fluorescent protein by a Ty1 tag.

To generate RED *T. brucei* parasites, the 1.68 Kb optimised version of the North American firefly *Photinus pyralis* luciferase (Branchini et al., 2005) was fused with a 30-bp sequence known as Ty1-tag (Bastin et al., 1996) and cloned into the pTSARib plasmid (Xong et al., 1998) by using *Xho*I and *Hin*dIII restriction enzymes to obtain the pTSARib-PpyRE9H-TY1 plasmid. Finally, the 1.4 Kb coding region of the tdTomato fluorescent protein was inserted downstream using *Hin*dIII and *Bam*HI. The resulting 8.9 Kb pTSARib-PpyRE9H-TY1-tdTomato vector, containing a blasticidin S resistance cassette, was linearised with *Sph*I to integrate the rDNA promoter locus.

In order to generate the RED *L. major* strain, the same chimeric PpyRE9H/TY1/tdTomato protein coding sequence was amplified by PCR from the pTSARib-PpyRE9H-TY1-tdTomato vector. PCR product was cut with *Bgl*II and *Not*I restriction enzymes and ligated into the same sites of the pLEXSY-hyg2 expression vector (Jena Bioscience GmbH, Germany).

2.3 Generation of the RED parasites

Parasites genetically engineered to express the chimeric multiplex reporter protein PpyRE9H/TY1/tdTomato in their cytosol will be further referred as RED strains. Trypanosomes BSF and PCF were transformed with an Amaxa Nucleofector (Lonza) (Burkard et al., 2007; MacGregor et al., 2013) and sub-cloned by limiting dilution. Clone selection was performed by measuring both bioluminescence in a microplate reader Infinite® 200 (Tecan) and fluorescence with a Muse® cell Analyzer (Merck-Millipore). Cells were routinely counted with an automated Muse® cell Analyzer (Merck-Millipore) and / or manually with a KOVA hemocytometer according to the manufacturer's recommendations.

L. major promastigote parasites were transformed with the large *Swa*I targeting fragment derived from the final 11.1 Kb pLEXSY-PpyRE9H-TY1-tdTomato plasmid by electroporation and subsequent plating on semisolid media containing 200 µg/ml hygromycin B as previously described (Kapler et al., 1990). The fluorescent and bioluminescent clones were confirmed by using a microplate reader Infinite® 200 (Tecan) and a Muse® cell Analyzer (Merck-Millipore).

2.4 In vitro fluorescence and bioluminescence quantification in RED parasites

To perform the parasite density / fluorescence-bioluminescence intensity assay with the RED PCF and BSF trypanosomes, as well as with promastigotes of the RED *L. major* strain, parasites were counted, centrifuged and resuspended in fresh SDM-G, HMI-11 and M199 medium, respectively. Then, 100µl (or 10^7 parasites) of this suspension were transferred into black clear-bottom 96-well plates and serial 2-fold dilutions were performed in triplicate adjusting the final volume to 200µl with 300 µg/ml of beetle luciferin (Promega, France). Luciferase activity was quantified after 10 minutes of incubation with a microplate reader Infinite® 200 (Tecan) or an IVIS® Spectrum imager (Perkin Elmer), following the instructions of the Promega Luciferase Assay System. After removal of intensity values from parasite-free medium corresponding to the background noise, results were analyzed as mean ±SD of three independent experiments.

2.5 Tsetse fly maintenance, infection by RED Trypanosoma brucei brucei *and dissection*

Glossina morsitans morsitans tsetse flies were maintained in Roubaud cages at 24°C and 60% hygrometry and fed through a silicone membrane with fresh mechanically defibrinated sheep blood. Teneral males (between 24h and 72h post-emergence) were allowed to ingest parasites in culture medium during their first meal through a silicone membrane. Here, we used either (i) cultured PCF trypanosomes at $5x10^6$ cells per millilitre in SDM79 medium supplemented with 10% foetal bovine serum, 8mM glycerol and 10mM glutathione (MacLeod

et al., 2007), or (ii) freshly *in vitro* differentiated stumpy BSF trypanosomes resuspended at $2x10^6$ cells per millilitre in SDMG medium supplemented with 10mM glutathione (MacLeod et al., 2007).

Flies were starved for at least 24 hours before being dissected. After rapid isolation of the salivary glands in a drop of phosphate buffer saline (PBS), whole tsetse alimentary tracts, from the distal part of the foregut to the malpighian tubules, were dissected and arranged lengthways in a another drop of PBS as previously described (Rotureau et al., 2011; Rotureau et al., 2012). Isolated organs were then either scrutinized under a microscope at high magnification and imaged, or individually dilacerated, dried on the slide and further treated for immuno-fluorescence (Rotureau et al., 2014).

Salivation tests were performed one month after the infective meal. First, cold-anesthetized flies were rapidly screened for the presence of a red fluorescent signal through the cuticle of their ventral abdomen under an epifluorescence M165FC stereomicroscope (Leica) at the 10x magnification. Positive flies were sorted, starved for 48h, and individual flies were allowed to probe on warm clean glass slides for 5 minutes. Saliva drops were eventually checked for the presence of fluorescent metacyclic trypanosomes under a DMIL LED epifluorescence microscope (Leica) at the x60 magnification.

2.6 Mouse strains and ethical statements

Wild type BALB/c and C57BL/6J mice from Janvier Laboratory were used as models for experimental infection and monitoring of the bioluminescence with IVIS Spectrum and IVIS CT. In addition, to allow for further 3D intravital imaging of the lymphatic and blood systems, C57BL/6J-Flk1-EGFP mice expressing a GFP-tagged *Kdr* (*Flk1*) gene encoding the vascular endothelial growth factor receptor 2 (VEGFR-2), were also used (Ema et al., 2006). This study was conducted in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the European Union (European Directive 2010/63/UE) and the French Government. The protocol was approved by the "Comité d'éthique en expérimentation animale de l'Institut Pasteur" CETEA 89 (Permit number: 2012-0043 and 2016-0017) and undertaken in compliance with Institut Pasteur Biosafety Committee (protocol CHSCT 12.131). BR is authorized to perform experiments on vertebrate animals (license #A-75-2035) and is responsible for all the experiments conducted personally or under his supervision.

2.7 Mouse infections with RED parasites

RED BSF (10^5 parasites/mouse) were used to inoculate seven-week-old male C57BL/6J or BALB/c mice (Janvier, France) by the IP route. Parasitemia was assayed daily by automated fluorescent cell counting with a Muse cytometer (Merck-Milllipore, detection limit at $5x10^2$

233 parasites/ml) according to the manufacturer's recommendations. Alternatively, mice were
234 infected via the natural route, i.e. by infective bites: tsetse flies with a mature salivary gland
235 infection, assessed by a salivation test, were individually allowed to bite on the belly of a
236 naïve mouse for about 15 minutes, and blood absorption was further verified by examination
237 of their abdomen.

238 Seven-week-old male BALB/c mice were injected with $1x10^6$ *L. major*
239 PpyRE9H/TY1/tdTomato stationary-phase promastigotes (4 to 5-day old) in the hind
240 footpads. After 4 weeks, animals were euthanized by cervical dislocation and popliteal lymph
241 nodes were dissected for imaging. The total number of living parasites invading the target
242 organs (popliteal lymph node draining the injected site) was calculated from serially-diluted
243 single-cell suspensions and dispensed into 96-well plates. Parasitic load expressed as
244 Limiting Dilution Assay Units (LDAU) after ten days of differentiation of amastigotes into
245 promastigotes. The number of parasites was calculated as follows: Limit Dilution Assay Units
246 (LDAU) = (geometric mean of titre from quadruplicate cultures) x (reciprocal fraction of the
247 homogenized organ added to the first well). The titre was the reciprocal of the last dilution in
248 which parasites were observed (Lima et al., 1997).

2.8 Ex vivo fluorescence imaging of RED parasites

250 For live fluorescence imaging of *Leishmania* promastigotes and all trypanosome stages,
251 parasites in blood, in culture medium or isolated from tsetse fly midgut were observed either
252 (i) with a DMI4000 microscope (Leica) and images were acquired with a Retiga-SRV camera
253 (Q-Imaging), or (ii) with a DMIL LED microscope (Leica) and images were acquired with a
254 digital color DFC450 C camera (Leica). Trypanosomes in tsetse organs were directly
255 monitored with a M165FC stereomicroscope (Leica) with a monochromatic DFC30000 G
256 camera (Leica). For intracellular *Leishmania* amastigote observations, peritoneal
257 macrophages were collected from one BALB/c mouse. Then, $5x10^5$ cells were plated on Ibidi
258 chambers and infected at a ratio of 10 stationary-phase promastigotes per macrophage as
259 previously described, and maintained for 72h at 37°C in 5% CO_2 (Spath et al., 2000).
260 Intracellular amastigotes were then observed with a DMI4000 microscope (Leica) and
261 images were acquired with a Retiga-SRV camera (Q-Imaging). For figure preparation,
262 specific ROI were selected and fluorescent frames were normalized to the same scale of
263 intensity with ImageJ (NIH).

2.9 In vivo fluorescence imaging of RED parasites

265 RED trypanosomes were monitored in the ear of Kdr (Flk1) C57BL/6J Rj mice by spinning-
266 disk confocal microscopy as described previously (Capewell et al., 2016). Briefly, mice were
267 first anaesthetised by IP injection of ketamine (Imalgene 1000 at 125 mg/kg) and xylazine

(Rompun 2% at 12.5 mg/kg). Mice were wrapped in a heating blanket and placed on an aluminium platform with a central round opening of 21 mm in diameter. A coverslip was taped on the central hole and the mouse was positioned so that the ear was lying on this oiled coverslip. Imaging was performed using an UltraView ERS spinning-disk confocal system (Perkin Elmer) with a x40 oil objective (1.3 numerical aperture). Movies were acquired by an EM-CCD camera (Hamamatsu) controlled by the Volocity software (Perkin Elmer) with an exposure time of 500 ms for a total of 30 to 120 s. Images were analysed using ImageJ 1.48v and its plugin Bio-formats importer (NIH).

2.10 In vivo bioluminescence imaging of RED parasites

Infection by bioluminescent parasites was monitored daily by detecting the bioluminescence in whole animals with the IVIS® Spectrum imager (Perkin Elmer). The equipment consists of a cooled charge-coupled camera mounted on a light-tight chamber with a nose cone delivery device to keep the mice anaesthetized during image acquisition with 1.5% isoflurane. D-luciferin potassium salt (Promega) stock solution was prepared in PBS at 33.33 mg/ml, filter-sterilized and stored in a -20°C freezer. To produce bioluminescence, mice were inoculated IP with 150 µl of D-luciferin stock solution (250mg/kg). After 10 minutes of incubation to allow substrate dissemination, all mice were anaesthetized in an oxygen-rich induction chamber with 2% isoflurane, and images were acquired by using automatic exposure (30 seconds to 5 minutes) depending on signal intensity. Images were analyzed with Living Image software version 4.3.1 (Perkin Elmer). Data were expressed in average radiance (p/s/cm²/sr) corresponding to the total flux of bioluminescent signal according to the selected area (total body of the mouse here). The background noise was removed by subtracting the bioluminescent / fluorescent signal of the control mouse from the infected ones for each acquisition.

Morphological / anatomical imaging technologies, such as computed tomography (CT) can be used to provide a combination of functional and anatomical data ensuring that maximum information is extracted from the infected animal model (Willmann et al., 2008). Longitudinal imaging is also used in pre-clinical studies to follow the progress of a disease or measure the effect of a therapeutic (Lim et al., 2011). Bioluminescence was acquired with a cooled CCD camera using the IVIS® Spectrum CT scanner (Perkin Elmer). Each animal was injected with 150 mg/kg of D-luciferin IP 10 min prior to imaging. Six spectrally resolved images were acquired using emission filters at 560, 580, 600, 620, 640, and 660 nm with a bandwidth of 20 nm each and an exposure time of 180 seconds. 3D tomographic reconstruction and superposition over the CT volume was performed using the DLIT algorithm available in the Living Image software 4.3. (Perkin Elmer), which uses single-view multispectral bioluminescence images to perform the reconstruction with segmentation of the CT images

in order to provide the mouse body boundary. The bioluminescence signal emitted by RED
AnTat 1.1E parasites was monitored 4 days after the inoculation of 10^5 BSF by IP route in
BALB/c mice.

2.11 Immuno-fluorescence analysis and immuno-histology

For immuno-histology, samples from the most bioluminescent skin regions were fixed in 4%
paraformaldehyde over-night, processed into paraffin blocks and trimmed. Sections were
processed for immuno-histochemical staining as described above. For immuno-fluorescence,
parasites were settled on poly-L-lysine coated slides and fixed in methanol at -20°C for 5
seconds and re-hydrated in PBS for 10 minutes. Slides were incubated with the BB2 primary
antibody diluted in PBS containing 0.1 % bovine serum albumin for 45 minutes at 37°C. The
BB2 monoclonal antibody targets the Ty1-tag (mouse IgG1, up to 1/10) (Bastin et al., 1996).
Slides were then washed and incubated with an anti-mouse-IgG1 secondary antibody
coupled to Cy3 (Jackson) for 30 minutes at 37°C. Slides were finally stained with 4',6-
diamidino-2-phenylindole (DAPI) for visualization of kinetoplast and nuclear DNA content,
and mounted under cover slips with ProLong antifade reagent (Invitrogen), as previously
described (Rotureau et al., 2011). Samples were observed either with a DMR microscope
(Leica) and images were captured with a CoolSnap HQ camera (Roper Scientific), or with a
DMI4000B microscope (Leica) and images were acquired with an ORCA-03G camera
(Hamamatsu). Image acquisition was controlled using Micro-manager (NIH) and images
were taken with the min/max threshold set at maximum.

2.12 Western blotting

For western blotting, samples were prepared by washing cells in serum-free medium before
dilution with PBS and boiling in Laemmli for 5 minutes. SDS-PAGE separation was carried
out after loading 10, 1 and 0.1 µg of total proteins per lane (corresponding to 10^6, 10^5 and
10^4 cells, respectively). Proteins were transferred onto polyvinylidene fluoride membranes for
7 minutes by using the Trans-Blot® Turbo™ technology (Bio-Rad), and blocked with 5%
skimmed milk in PBS with 0.1% Tween 20 for 1 h prior to probing. The following primary
antibodies were used by dilution in 2% milk in PBS with 0.1% Tween 20: L8C4 (non-diluted)
(Kohl et al., 1999) and BB2 (1:50) (Bastin et al., 1996). Anti-mouse horseradish peroxidase-
conjugated secondary antibodies (Amersham Biosciences) were used at a 1:20,000 dilution.
Membranes were washed in PBS with 0.1% Tween 20 after blocking, between probing with
primary and secondary antibodies and prior to band detection by chemiluminescence (GE
healthcare).

2.13 Measurements, normalization and statistical analysis

Normalization of signals was carried out by parallel manipulation of min/max signals in ImageJ (NIH). For clarity purposes, brightness and contrast of several pictures were adjusted after their analysis in accordance with editorial policies. Statistical analyses and plots were performed with XLSTAT 2015.4.01 (Addinsoft) and Excel 2011 (Microsoft), respectively. Statistical analyses include linear regression analyses for bioluminescence / fluorescence intensity Vs. parasite density, ANOVA for growth curves, and ANCOVA for fly infection rates, with Tukey ad-hoc post-tests for inter-group comparison, all with 95% confidence.

3. Results

We have generated a new chimeric triple reporter recombinant protein of 1,036 aa named PpyRE9H/TY1/tdTomato and composed by the fusion of (i) the human codon-optimized red-shifted version of the North American firefly *Photinus pyralis* luciferase (Branchini et al., 2005), (ii) a short amino acid sequence known as Ty1-tag (Bastin et al., 1996), and (iii) the tdTomato fluorescent protein (Fig. S1).

During the plasmid-construct amplification in *E. coli* DH5α, the fusion protein expression was attested by the red colour of bacterial pellets and further confirmed under an epifluorescence microscope, demonstrating the potential usefulness of this construct also in prokaryotic cells. The triple reporter construct was then expressed in the cytoplasm of the eukaryotic protist parasites *Leishmania major* and *Trypanosoma brucei brucei.* These strains will be further referred to as the RED parasites in the entire article.

3.1 In vitro and in vivo applications with RED L. major parasites

In order to explore the usefulness of the novel triple reporter construct, a transgenic *Leishmania major* strain expressing the PpyRE9H/TY1/tdTomato was generated. Promastigotes of *L. major* Friedlin reference strain were electroporated and selected in semi-solid plates to obtain single fluorescent and bioluminescent clones. The presence of the construct was checked by PCR on genomic DNA (Fig. S2A-B). Parasites were scrutinized under an epifluorescence microscope and their fluorescence was compared to that of wild type cells (WT) (Fig. 1A-B). A strong and specific red fluorescent signal was homogenously distributed in both the cytoplasm and the flagellum of RED promastigote cells only, but it was absent from their nucleus. The fluorescence of RED cells was further confirmed at the population level by flow cytometry analysis (Fig. 1C-D). Moreover, parasite proliferation *in vitro* was comparable to that of the parental cell line (Fig. 1E).

A linear correlation was found between the number of cells and the emission of bioluminescence, by measuring the signal *in vitro* in 96-well plates with an IVIS Spectrum imager (Fig. 2A), being able to detect as few as 10^3 parasites (Fig. 2B). In parallel, this linear correlation was verified in a TECAN multiwell-plate reader for 5 RED sub-clones (Fig. 2C). Similarly, a linear correlation between the fluorescent signal intensity and the number of parasites was observed with both the imager (Fig. 2D), with a limit of detection at 10^4 cells (Fig. 2E), and the plate reader for 5 RED sub-clones (Fig. 2F).

To test the efficiency of the RED *L. major in vivo*, the virulence of the transfected parasites was firstly recovered by passing the strain once through BALB/c mice. In order to determine whether RED *L. major* parasites could be detected *in vivo* using whole-body imaging, 10^6 stationary-phase parasites were injected subcutaneously into the hind footpads of BALB/c

mice. Lesion progression was monitored both by the direct measurement of the bioluminescence (Fig. 3A) and fluorescence (Fig. 3B) emitted by RED amastigotes recorded in the IVIS Spectrum, and by the follow-up of the hind-limb lesions over four weeks (Fig. 3C-D). The bioluminescent signal start to increase from 5 days post-infection up to the end of the experiment, reaching a maximum radiance of 6.3×10^6 p/s/cm^2/sr after four weeks of infection. However, *in vivo* follow-up of the fluorescence during the infection course showed that the fluorescent signal was visible only from the 3rd week after the infection, leading to the expected conclusion that bioluminescence was much more sensitive to evaluate the progression of infection in this context (Fig. 3C-D). After 29 days, mice were sacrificed and the popliteal lymph nodes were extracted and imaged (Fig. 3E-F). Parasitic load (LDAU) in the popliteal lymph node draining the lesion of infected animals, determined by the limiting dilution method, was 4.8×10^6 as an average of both infected organs per mouse. These results are in accordance with regular parasitic load experiments.

Then, *in vitro* infection was performed in freshly collected resident peritoneal macrophages in order to allow parasites to differentiate into intracellular amastigotes forms in which the expression of the triple marker was confirmed as assessed by the presence of a specific red fluorescent signal (Fig. S3A).

3.2 In vitro uses of RED T. brucei parasites

In order to explore the usefulness of the novel triple reporter construct in another trypanosomatid parasite, transgenic *Trypanosoma brucei brucei* mammalian bloodstream forms (BSF) and insect procyclic forms (PCF) were transformed with the pTSARib-PpyRE9H-TY1-tdTomato vector and immediately sub-cloned by limiting dilution. The presence of the construct was checked by PCR on genomic DNA (Fig. S2). RED PCF trypanosomes in culture were scrutinized under a fluorescence microscope for a direct visual confirmation of their fluorescent phenotype as compared to WT cells. As expected, the triple reporter was homogenously distributed in the cytoplasm, and in a lesser extend in the flagellum, only in transformed parasites, but it was absent from their nucleus (Fig. 4A-B). The fluorescence of RED cells was further confirmed at the population level by flow cytometry analysis (Fig. 4C-D). Similar observations were done with RED BSF trypanosomes, yet with a lower overall fluorescence intensity as compared to that in RED PCF cells (Fig. 4E-H). Although the proliferation of PCF was significantly slower in transformed parasites compared to WT cells (doubling time of 14.5 h and 10.5 h, respectively, ANOVA p=0.034) (Fig. 4I), the BSF proliferation rate *in vitro* was comparable to that of the parental cell line (doubling time of 6.5 h for both) (Fig. 4J).

First, in order to verify the reliability of the bioluminescent marker as well as to define its detection threshold, a parasite density / bioluminescence intensity analysis was performed in

14

417   96-micro-well plates with an IVIS Spectrum imager (Fig. 5A-B). Parasite density and
418   bioluminescence intensity were correlated when bioluminescence levels were higher than
419   10^4 p/s/cm²/sr, corresponding to about 10^2 RED BSF or RED PCF parasites, allowing
420   estimation of the parasite density from *in vivo* imaging over this threshold (Fig. 5C). In
421   parallel, this linear correlation was verified in a TECAN multiwell-plate reader for several
422   RED sub-clones of both parasite stages (Fig. 5D-E), yet with some bioluminescence intensity
423   level variations between clones, especially for RED PCF. Second, the fluorescent signal
424   emitted by these RED *T. brucei* parasites was measured in a IVIS fluorescence reader and
425   appeared to be correlated to their number in a dynamic range starting from 10^3 PCF and 10^4
426   BSF (Fig. 5F-G), although the overall fluorescence intensity level in RED BSF was much
427   lower compared to that in RED PCF (Fig. 5H). This is possibly due to the existence of BSF-
428   specific chemical conditions in the cytosol, such as pH variations and / or high localized
429   concentrations of cations, that may affect tdTomato activity, or to a higher photobleaching
430   rate at 37°C, rather than to a lower turn-over or to a reduced folding efficiency, since the
431   luciferase activity was not affected, as assessed by high bioluminescence levels. This
432   observation was further confirmed by testing several subclones of both stages in a plate
433   reader (Fig. 5I-J).

434   ***3.3 In vivo uses of RED T. brucei parasites***

435   To test whether RED trypanosomes could be monitored *in vivo* in their insect vector,
436   *Glossina morsitans morsitans* teneral males were experimentally infected with RED PCF
437   parasites through a silicone membrane. One month after the infective meal, flies were
438   screened under an epifluorescence binocular microscope for the presence of a specific red
439   fluorescence through their abdominal cuticle, reflecting the presence of RED parasites in
440   their midgut (Fig. 6A-B). To assess the stability of the triple reporter expression during the
441   course of the infectious process, i.e. without any selective antibiotic pressure, both negative
442   and positive flies were dissected and the presence of parasites in their midgut was verified
443   by both light and epifluorescence microscope examination at a higher magnification (Fig. 6C-
444   E). Living parasites were only detected in flies that were initially scored positive by
445   fluorescence examination of the intact abdomen, demonstrating the stability of the triple
446   reporter *in vivo* (Fig. 6F and Movie1). It is noteworthy that the red fluorescent component of
447   the triple reporter could also be used to directly scrutinize the parasite development, motility
448   and interactions *in vivo* or *ex vivo* within the tsetse host tissues and organs (Fig. 6E-F and
449   Movie1) (Schuster et al., 2017). Then, in order to assess the virulence of RED parasites in
450   the tsetse fly, a total of 17 batches of flies were fed with either WT BSF, WT PCF, RED BSF
451   or RED PCF parasites in culture. Experimental infections were performed over a one-year
452   period by comparing strains at least two by two. In total, 606 flies were dissected after one

month, and the infection rates in the midgut and salivary glands were compared between groups (Fig. 6G). For WT parasites, fly infection by BSF gave rise to significantly higher infection rates, especially in the midgut (p=0.019, ANOVA's Tukey ad-hoc post-test, 95% confidence). In total, RED parasites produced similar infection rates as compared to WT controls, except for the RED BSF that were significantly less prone than WT BSF to produce mature infection in the salivary glands (p=0.003, ANOVA's Tukey ad-hoc post-test, 95% confidence).

Conveniently, the possibility of assessing the infection by a simple observation of living flies allowed us to anticipate the number of flies which could be potentially infected at the salivary gland level. Therefore, in another experiment, only fluorescent flies were used for salivation tests one month after the infective meal in order to sort living tsetse flies with a mature salivary gland infection for further natural transmission experiments. Single flies with salivary gland infection were then used to naturally infect mice, and the course of the infection was monitored daily by acquiring the bioluminescent signal emitted by the parasites in the entire organism with an IVIS Spectrum imager (Fig. 7A). Mice bitten by uninfected flies and not bitten at all were used as controls. The parasitemia was also daily quantified by detecting RED fluorescent BSF in 5 µl blood samples with a Muse bench flow-cytometer. In parallel, the parasitemia was verified by manual counting of living parasites with a KOVA hemocytometer (Fig. 7B). Similar parasitemia quantifications were obtained by both methods (not shown), demonstrating that the fluorescence, although being limited in RED BSF as compared to RED PCF, was stably maintained over the course of the infection. A significant bioluminescent signal was detected as soon as after 1 to 2 days and was persisting until the end of the experiment, whereas the parasitemia was only first detected after 17 days and further fluctuated by characteristic waves (Fig. 7C). This demonstrates that a substantial population of parasites remained extravascular during the entire course of the infection. As assessed by ventral and dorsal bioluminescence imaging, both the parasite distribution and density evolved in space and time during the entire infection course (Fig. 7C). This experiment reproduces our previous study where the bioluminescent signal intensity was used for quantifying the correlated parasite densities (Capewell et al., 2016). In the latter study, mice infected with RED BSF were sacrificed and dissected to record the bioluminescent signal in isolated organs: in contrast to the bioluminescent signals detected in the spleen, the liver, the inguinal and cervical lymph nodes and some adipose tissues, the skin was found to constitute the major anatomical reservoir of parasites even in the absence of detectable parasites in the blood (Capewell et al., 2016). Alternatively, in order to study the differential anatomical distribution of the RED parasites in living mice, we attempted to test longitudinal imaging with the IVIS Spectrum CT imager. The computed tomography application allowed us to extract the spatial 3D-localization of RED parasites, although only

490   in heavily infected organs (spleen, liver and lymph nodes) (Fig. 7D). To refine the
491   observation of living parasites developing in the skin at the cell level, the same naturally-
492   transmitted RED trypanosomes were also imaged *in vivo* using a spinning-disk confocal
493   microscopy setting in the C57BL/6J-Flk1-EGFP mouse line that present green fluorescent
494   endothelial cells in the lymphatic and blood vessels (Ema et al., 2006). Strikingly,
495   extravascular trypanosomes were observed gliding and dividing in the dermal layer of the ear
496   (Fig. S3B) (Capewell et al., 2016).
497   In total, the RED trypanosomes were comparable to the parental WT strain in terms of
498   pleomorphism and tsetse infectivity. Both red markers were stably expressed in all stages of
499   the parasite cycle, both *in vivo* and *in vitro*, in cells kept from 27°C to 37°C, in the absence of
500   any antibiotic pressure, and over at least 3 months in total. All these features allowed us to
501   scrutinize the kinetics of the same infection at different scales *in vivo* (single cells, tissue
502   level and whole body).

503   ### *3.4 Immuno-detection of the triple reporter construct with the TY1 tag*

504   The bioluminescent and fluorescent properties of the two reporter proteins used in the
505   construct mainly rely on their conformation and on the physico-chemical nature of the micro-
506   environment. For this reason, common regular protocols, such as for electron microscopy,
507   histology or protein biochemistry are known to at least partially alter protein structures, which
508   results in a loss of enzymatic activity, fluorescence emission or immune-specificity. For these
509   reasons, we have introduced the small Ty1-tag between the red-shifted luciferase and the
510   tdTomato in order to stabilize the fusion protein and to serve as a third reporter in specific
511   conditions where the two other reporters would not be detected. The Ty1-tag can be
512   recognized by two different monoclonal antibodies with overlapping binding sites: BB2, which
513   is an immunoglobulin G (IgG1) and TYG5, which is an immunoglobulin A.
514   The expression and accessibility of the Ty1 epitope was first assessed by
515   immunofluorescence staining with BB2 in both RED *T. brucei* PCF (Fig. 8A) and RED *L.*
516   *major* promastigotes (Fig. 8B) after a 5-seconds methanol fixation. The homogenous
517   distribution of the signal within the entire cytoplasm and flagellum was similar to that obtained
518   directly by recording the tdTomato emission in living cells (Fig.1B and 4B). Then, we
519   performed a western blot in denaturing conditions in order to test the ability of BB2 to detect
520   the Ty1 reporter in total protein samples from RED *T. brucei* PCF and RED *L. major*
521   promastigote parasites (Fig. 8C). Different amounts of parasites (10^6, 10^5 and 10^4) were
522   tested in order to confirm the correlation between the total amount of proteins and the
523   detection of the tag. A specific band of 110 kDa was revealed only in the protein samples
524   from 10^5 and 10^6 transformed parasites. Finally, highly bioluminescent regions of the skin of
525   naturally infected mice (Fig. 7A) were sampled, fixed in paraformaldehyde, paraffin-

embedded and treated for immuno-histological analysis with BB2. Although no direct red fluorescence was detected, probably as the result of the sample heating for paraffin-embedment, the BB2 antibody was able to *in situ* label skin-dwelling-parasites expressing the Ty1 tag (Fig. 8D). These results confirm the potential use of the Ty1-tag included in the chimeric triple reporter fusion protein in a wide variety of immunological assays pre-requiring chemical denaturation and / or heating of the samples.

4 Discussion and conclusion

4.1 Importance of intravital imaging in research on trypanosomatids

Intravital imaging approaches already led to important discoveries on the biology of trypanosomatid parasites. The use of bioluminescent *T. brucei* parasites has successively enable different research groups to identify specific anatomical niches that strongly support the preponderance of extravascular parasite populations during the infectious process. Claes and co-workers showed that parasites located in the testis were not cleared by trypanocidal drugs, as they were protected by the blood-testis barrier (Claes et al., 2009). Trindade *et al.* and Caljon *et al.* further observed a close association of *T. b. brucei* with adipocytes in fat tissues and subcutaneous adipose layers, respectively (Caljon et al., 2016; Trindade et al., 2016). At the same time, together with colleagues from Glasgow, Conakry and Kinshasa, we have demonstrated that the majority of *T. b. gambiense* parasites were actually rather developing in the extravascular matrix of the dermis of murine models, where they were seen actively swimming and dividing (Capewell et al., 2016). In another study by D'Archivio and co-workers, the importance of the late brain invasion by *T. vivax* parasites was assessed in a dynamic manner by intravital imaging of a bioluminescent strain (D'Archivio et al., 2013). Recently, the principal clinical, immunological and parasitological features associated with visceral leishmaniasis were characterized by *in vivo* imaging approaches of *L. donovani* transgenic parasites in a golden hamster model (Rouault et al., 2017) and the occurrence of multiple phases of brain invasion and inflammation was further unambiguously demonstrated (Melo et al., 2017). Bioluminescence has also been recently used to monitor the evolution of the *T. cruzi* parasite load in the digestive tract of its triatomine vector *Rhodnius prolixus* (Dias Fde et al., 2015).

Applied to drug development in high-throughput (HTS) *in vitro* assays as well as in *in vivo* murine models, bioluminescent and fluorescent trypanosomatid reporter strains have also proved their usefulness. Intravital multi-photon microscopy revealed that diminazene was unable to target *T. b. brucei* parasites that have already crossed the blood-brain barrier three weeks after the infection in a mouse model of stage 2 sleeping sickness (Myburgh et al., 2013). Reimão and co-workers evaluated the efficacy of miltefosine *in vivo* in a luciferase-

expressing *L. infantum chagasi* experimental model of infection in hamsters that mimics human disease (Reimao et al., 2015). A non-invasive model of lesion suppression has been recently validated for the semi-HTS screening of compounds against cutaneous leishmaniasis by using a bioluminescent strain of *L. major* (Caridha et al., 2017). Other studies have taken advantage of the use of fluorescent and / or bioluminescent parasites to screen myriads of compounds in multi-well plates. Annang and co-workers validated a HTS platform to screen a subset of microbial extracts against the 3 trypanosomatid-borne diseases in 384-well plates by using, among them, bioluminescent-*L. donovani* parasites (Annang et al., 2015). The use of *ex vivo* cultures from infected tissues or organs has been explored and exploited as well by some other research groups with bioluminescent *L. donovani* (Osorio et al., 2011) and *L. major* (Peniche et al., 2014), as well as with infrared-emitting *L. infantum* parasites (Calvo-Alvarez et al., 2015b).

4.2 Novel perspectives offered by the triple reporter

Most of these studies are relying on the use of constructs encoding for single green fluorescent probes or single luciferases emitting in the green. This is not optimal for intravital imaging due to the high absorption of the light by the tissue components at these wavelengths, and this also limits the monitoring to a single scale, i.e. whole organism or tissue / cell level. To bypass this technical bias, we have simultaneously used two reporters stably producing light in the red wavelengths with a reduced absorption by tissues, resulted in an enhanced sensitivity of the monitoring, with equivalent detection thresholds compared to previous studies (Burrell-Saward et al., 2015; McLatchie et al., 2013; Taheri et al., 2015; Van Reet et al., 2014). In the present study, as in the one of McLatchie and colleagues (McLatchie et al., 2013), we used a human codon optimized *PpyRE9h* sequence that was adapted to *T. brucei*. However, although this would theoretically have not been optimal for *Leishmania,* whose genome are more GC-rich, our results demonstrate that this actually doesn't seem to affect the triple marker expression. Taheri and collaborators have monitored and tracked a cutaneous *Leishmania* infection in real time, by generating stably-transgenic *L. major* parasites that expressed a bi-reporter protein constituted by an EGFP fused to a firefly luciferase. They demonstrated that the simultaneous use of two reporters increases the experimental sensitivity for detection and quantification of parasites *in vitro* as well as during real-time infection in mice (Taheri et al., 2015). In the present work, the constitutive cytosolic expression of the red triple reporter in African trypanosome and *Leishmania* strains in mice allowed us to study the kinetics of the infection in the same animal and at different scales by successively using a combination of complementary imaging technologies. We have demonstrated the correct expression of the transgenic constructs with a clear correlation between the number of parasites and the bioluminescent / fluorescent signals *in vitro*, thus

permitting the estimation of the parasite load by means of signal acquisition. However, using this methodology for an exact *in vivo* quantification of the parasite densities in experimental host tissues will further require a higher degree of standardization and some more controls in order to obtain reliable and reproducible quantitative results. In total, the use of this triple reporter led us to access both qualitative and quantitative data on the development of RED *L. major* in a mouse model, as well as on the full parasite cycle of RED *T. brucei* in the tsetse digestive tract and in the mammalian host after natural transmission. Furthermore, the Ty1-tag was also used to refine the analysis of the same biological material after intravital imaging, by immuno-based approaches (western blot, immunofluorescence analyses, immunohistochemistry).

It was the powerful combination of both fluorescence and bioluminescence imaging that allowed us to recently formally prove that skin-dwelling parasites were readily ingested by tsetse flies to fulfil their parasite cycle (Capewell et al., 2016). In the context of other HAT research projects, the optimised triple reporter line described here would also possibly enhance the application of *in vivo* imaging to study stage II African trypanosomiasis in murine models. Indeed, it will provide a new framework for investigating host-parasite relationships, particularly in the context of CNS infections. In addition, it should be ideally suited to drug evaluation programmes. This RED triple reporter construct represents a powerful tool that could also be useful in many other fields of research where cellular model can be transformed. According to the plasmid vector used and to its way of integration, the signal intensities could theoretically be modulated. Moreover, although the size of the triple reporter is not exactly appropriated, one could think about its potential use for protein tagging. Possible simultaneous qualitative and quantitative applications in research are the use of its bioluminescence component for *in vitro* HTS drugs screening and *in vivo* monitoring (microbiology, immunology, parasitology, oncology, pharmacology, vaccinology…), the use of its fluorescence for *in vitro* assays (HTS drug screening, investigation of biological processes...) and *in vivo* monitoring (microbiology, immunology, parasitology, oncology…), and the use of the Ty1-tag for immuno-detection in fixed and treated samples after real-time intravital imaging.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution statement

ECA, CCT and AC contributed to the experiments.

ECA and BR contributed to study design, data analysis and manuscript writing.

Figure legends

649 **Fig. 1: Fluorescence pattern and growth of the RED *L. major*.**

650 (A-B) Live epifluorescence microscopy images of a WT *L. major* promastigote (A) and a RED

651 *L. major* promastigote (B) (normalized TdTomato fluorescence in red and DNA staining in

652 blue). (C-D) Ungated flow-cytometry analysis profiles confirming the non-fluorescence and

653 red fluorescence (in arbitrary units) of WT (C) and RED (D) *L. major* promastigote

654 populations, respectively. (E) Growth profiles (mean ± SD from triplicates) of RED *L. major*

655 promastigotes (in red) compared to their parental WT strain (in blue).

656 **Fig. 2: Quantification of the RED *L. major* bioluminescence and fluorescence.**

657 Images of the bioluminescent (A) and fluorescent (D) signals emitted by serial 1/2 dilutions of

658 RED *L. major* promastigotes observed with an IVIS Spectrum in a 96-well plate. The same

659 analysis was performed in triplicates for the RED strain (lines RED 1 to RED3). An equivalent

660 serial dilution of WT promastigotes was used as control (line WT). The last three wells

661 containing only medium were used as blank (red square). Radiance intensity scales are

662 provided in $p/s/cm^2/sr$ on the right of each picture. (B, E) The mean bioluminescent (B) and

663 fluorescent (E) signals (± SD) emitted by RED promastigotes show a significant linear

664 correlation with the number of cells (linear regression analysis; r^2=1.0 with p<0.0001 for BL;

665 r^2=0.99 with p<0.0001 for fluorescence). (C, F) Bioluminescent (C) and fluorescent (F)

666 signals (± SD) emitted by 5 different RED *L. major* clones were measured in triplicates with a

667 TECAN plate reader and plotted in arbitrary units (AU) according to the parasite density. A

668 similar linear correlation was observed for all sub-clones.

669 **Fig. 3: *In vivo* imaging applications with the RED *L. major*.**

670 (A-B) *In vivo* whole-body images of BALB/c mice, including 1 uninfected control (C) and 4

671 infected mice (1 to 4), acquired with an IVIS Spectrum imager. Bioluminescent (A) and

672 fluorescent (B) signals acquired on the hind footpads 28 days post-infection. Radiance

673 intensity scales are provided in $p/s/cm^2/sr$ under each picture. (C, D) Bioluminescent (C) and

674 fluorescent (D) signals on the hind footpads were measured and plotted over the course of

675 the infection. (E, F) RED *L. major* amastigotes residing in the popliteal draining lymph nodes

676 of 2 mice dissected 4 weeks after inoculation emit bioluminescence (E) and fluorescence (F).

677 Radiance intensity scales are provided in $p/s/cm^2/sr$ under each picture.

678 **Fig. 4: Fluorescence pattern and growth of the RED *T. brucei* PCF and BSF stages.**

679 (A-B and E-F) Live epifluorescence microscopy images of WT (A and E) and RED (B and F)

680 *T. brucei* PCF (A-B) and BSF (E-F) (normalized TdTomato fluorescence in red and DNA

681 staining in blue). (C-D and G-H) Ungated flow-cytometry analysis profiles confirming the non-

fluorescence and red fluorescence (in arbitrary unit) of WT (C and G) and RED (D and H) *T. brucei* PCF (C-D) and BSF (G-H) populations, respectively. (I-J) Growth profiles (mean ± SD from triplicates) of WT (in blue) and RED (in red) *T. brucei* PCF (I) and BSF (J).

Fig. 5: Bioluminescence and fluorescence quantification in the RED *T. brucei* PCF and BSF stages.

Images of the bioluminescent (A-B) and fluorescent (F-G) signals emitted by serial 1/2 dilutions of RED *T. b. brucei* PCF (A and F) and BSF (B and G) observed with an IVIS Spectrum in 96-well plates. The same analyses were performed in triplicates for the RED strains (lines RED 1 to RED3). An equivalent serial dilution of WT parasites was used as control (lines WT). The last three wells containing only medium were used as blank (red square). Radiance intensity scales are provided in $p/s/cm^2/sr$ on the right of each picture. (C and H) The mean bioluminescent (C) and fluorescent (H) signals (± SD) emitted by RED PCF (in blue, linear regression analysis with $p<0.0001$, $r^2=0.97$ for BL and $r^2=0.96$ for fluorescence) and RED BSF (in red, linear regression analysis with $p<0.0001$, $r^2=0.87$ for BL and $r^2=0.97$ for fluorescence) show a significant linear correlation with the number of cells. (D-E and I-J) Bioluminescent (D-E) and fluorescent (I-J) signals (± SD) emitted by 3 different RED *T. b. brucei* PCF sub-clones (D and I) and 4 different RED *T. b. brucei* BSF subclones (E and J) were measured in triplicates with a TECAN plate reader and plotted in arbitrary units (AU) according to the parasite density. A similar linear correlation was observed for all sub-clones.

Fig. 6: Imaging applications with the RED *T. brucei* in the tsetse fly host.

Glossina morsitans morsitans teneral males were fed with RED PCF and observed under an epifluorescence binocular microscope after one month in order to sort infected flies. A strong red fluorescent signal was observed through the ventral abdomen of infected flies (B) but not in non-infected flies (A). This was confirmed by dissection and observation of the fly midgut (C and D, respectively). Fluorescence was even detected dorsally in strongly infected flies (E), and individual RED parasites could be distinguished under the epifluorescence binocular microscope in and outside dissected midguts (F, still image from movie S1). (G) The virulence of RED parasites was assessed by feeding batches of tsetse flies with either WT BSF, WT PCF, RED BSF or RED PCF parasites resuspended in SDMG culture medium. The histogram shows the infection rates in the midgut and salivary glands observed one month post infection (* statistically significant difference between indicated groups with $p<0.02$ by ANCOVA's Tukey ad-hoc post-test at 95% confidence).

Fig. 7: Imaging applications with the RED *T. brucei* in the mammalian host after natural transmission.

(A) *In vivo* whole-body images of C57BL/6J mice acquired with an IVIS Spectrum imager 8, 15 and 22 days after a tsetse fly bite, including one mouse bitten an infected tsetse fly (+), one bitten by a non-infected fly (-) and one non-bitten (0). Radiance intensity scales are provided in $p/s/cm^2/sr$. (B) Live RED BSF in a blood sample from mouse + (DNA in blue). (C) Total ventral (blue squares) and dorsal (green triangles) bioluminescent signals (total radiance in $p/s/cm^2/sr$) were measured, normalized to negative controls and plotted over the course of the infection together with the parasitemia (red dots) for mouse +. (D) Bioluminescence and anatomical 3D computed tomography images acquired by an IVIS Spectrum CT on a BALB/c mouse 29 days post-inoculation. The 3D reconstruction view on the right panel has been decomposed in pictures along 3 distinct plans showed on the left (coronal in red, sagittal in blue and transaxial in green).

Fig. 8: Detection of RED trypanosomatids with the Ty1-tag after sample alteration by specific treatments.

(A, B) Immunofluorescence images of methanol-fixed RED *T. brucei* PCF (A) and RED *L. major* promastigotes (B) stained with the BB2 antibody (Ty1 in red) and DAPI (DNA in blue). (C) Reactivity of the BB2 monoclonal antibody against the Ty1-tag epitope (110 kDa) by Western Blot analysis. The L8C4 anti-PFR2 antibody (69 kDa) was used as a loading control. Total protein extracts from 10^6, 10^5 and 10^4 cells were used to compare the expression levels of the Ty1-tag in RED *T. brucei* procyclic forms and RED *L. major* compared to their respective WT control strains. Ladders (L) are provided on both sides of the blot with their respective size in kDa. (D) Immuno-histological analysis of a paraffin-embedded bioluminescent skin section from a mouse infected with RED *T. brucei*, *in situ* stained with the BB2 antibody (Ty-1 in red) and DAPI (DNA in blue). A skin-dwelling-parasite expressing the Ty1-tag situated in the extracellular matrix of the dermis is indicated with a white arrow.

Supplementary material

743 **Fig. S1: The PpyRE9/TY1/tdTomato fusion protein.**

744 *(A)* Schematic view of the protein regions (1,036 aa). (B) DNA sequence (3,111 bp).

745 **Fig. S2: Detection of the *PpyRE9H/TY1/tdTomato*-encoding gene and integration**
746 **strategies into genomic *RRNA* loci of *T. brucei* and *L. major*.**

747 (A) PCR analysis for confirmation of the genomic presence of the triple reporter sequence
748 (primers 1/2) and the specific antibiotic cassettes in RED *T.brucei* (primers 3/4) and RED *L.*
749 *major* (primers 5/6) parasites. (B) The upper scheme shows the final structure of the 18S
750 *RRNA* locus of *T. brucei* with the planned integration of the triple marker sequence. The
751 bottom scheme represents the expected integration of the chimeric 3.1 Kb multiplex reporter
752 protein PpyRE9H/TY1/tdTomato in the genome of *L. major*. PARP SL: procyclin splice site;
753 bsd: blasticidin resistance cassette; utr1: 5′ untranslated region of *aprt* gene; utr2: 1.4 kb
754 intergenic region from *cam* operon; and utr3: 5′ UTR of *dhfr-ts* gene; hyg: hygromycin B
755 resistance cassette. Primers 1 and 2 amplify the 3.1 Kb region of the PpyRE9H-TY1-
756 tdTomato sequence (1-GGAGGACGCCAAGAACATCAAG; 2-
757 TTACTTGTACAGCTCGTCCATGC); primers 3 and 4 amplify the blasticidin antibiotic
758 cassette (3-ATGGCCAAGCCTTTGTCTCAAG; 4-TTAGCCCTCCCACACATAACCAG); and
759 primers 5 and 6 were used to amplify the hygromycin resistance cassette (5-
760 ATGAAAAAGCCTGAACTCACCGC; 6-CTATTCCTTTGCCCTCGGACG).

761 **Fig. S3: Further imaging applications with RED parasites to monitor host cell / parasite**
762 **interactions at the cellular level.**

763 (A) RED *L. major* intracellular amastigotes within BALB/c peritoneal macrophages after 72 h
764 of incubation (DNA in blue and red fluorescent amastigotes in red). (B) Still-image from a
765 spinning-disk confocal microscope movie showing extravascular RED *T. brucei* parasites (in
766 red) in the surrounding of a vascular or lymphatic vessel (in green) in the ear dermis of a
767 living C57BL/6J-Flk1-EGFP mouse 21 days after an infective bite.

768 **Movie S1: RED *T. b. brucei* in the posterior midgut of a tsetse fly 7 days after the**
769 **infective meal.**

770 Living RED *T. b. brucei* procyclic parasites observed with a binocular epifluorescence
771 microscope within the posterior midgut of an infected tsetse fly 7 days after the infectious
772 meal. Red fluorescent parasites can be seen swimming outside the organ.

References

Aksoy, S., Buscher, P., Lehane, M., Solano, P., Van Den Abbeele, J., 2017. Human African trypanosomiasis control: Achievements and challenges. PLoS Negl Trop Dis 11, e0005454.

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., Team, W.H.O.L.C., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671.

Annang, F., Perez-Moreno, G., Garcia-Hernandez, R., Cordon-Obras, C., Martin, J., Tormo, J.R., Rodriguez, L., de Pedro, N., Gomez-Perez, V., Valente, M., Reyes, F., Genilloud, O., Vicente, F., Castanys, S., Ruiz-Perez, L.M., Navarro, M., Gamarro, F., Gonzalez-Pacanowska, D., 2015. High-throughput screening platform for natural product-based drug discovery against 3 neglected tropical diseases: human African trypanosomiasis, leishmaniasis, and Chagas disease. J Biomol Screen 20, 82-91.

Barquilla, A., Saldivia, M., Diaz, R., Bart, J.M., Vidal, I., Calvo, E., Hall, M.N., Navarro, M., 2012. Third target of rapamycin complex negatively regulates development of quiescence in Trypanosoma brucei. Proc Natl Acad Sci U S A 109, 14399-14404.

Bastin, P., Bagherzadeh, Z., Matthews, K.R., Gull, K., 1996. A novel epitope tag system to study protein targeting and organelle biogenesis in Trypanosoma brucei. Mol Biochem Parasitol 77, 235-239.

Beattie, L., Kaye, P.M., 2011. Leishmania-host interactions: what has imaging taught us? Cell Microbiol 13, 1659-1667.

Branchini, B.R., Southworth, T.L., Khattak, N.F., Michelini, E., Roda, A., 2005. Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications. Anal Biochem 345, 140-148.

Brookman, J.L., Stott, A.J., Cheeseman, P.J., Burns, N.R., Adams, S.E., Kingsman, A.J., Gull, K., 1995. An immunological analysis of Ty1 virus-like particle structure. Virology 207, 59-67.

Brun, R., Schonenberger, 1979. Cultivation and in vitro cloning or procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Short communication. Acta Trop 36, 289-292.

Burkard, G., Fragoso, C.M., Roditi, I., 2007. Highly efficient stable transformation of bloodstream forms of Trypanosoma brucei. Mol Biochem Parasitol 153, 220-223.

Burrell-Saward, H., Rodgers, J., Bradley, B., Croft, S.L., Ward, T.H., 2015. A sensitive and reproducible in vivo imaging mouse model for evaluation of drugs against late-stage human African trypanosomiasis. The Journal of antimicrobial chemotherapy 70, 510-517.

Buscher, P., Cecchi, G., Jamonneau, V., Priotto, G., 2017. Human African trypanosomiasis. Lancet.

809   Caljon, G., Van Reet, N., De Trez, C., Vermeersch, M., Perez-Morga, D., Van Den Abbeele,
810   J., 2016. The Dermis as a Delivery Site of Trypanosoma brucei for Tsetse Flies. PLoS
811   Pathog 12, e1005744.

812   Calvo-Alvarez, E., Alvarez-Velilla, R., Fernandez-Prada, C., Balana-Fouce, R., Reguera,
813   R.M., 2015a. Trypanosomatids see the light: recent advances in bioimaging research. Drug
814   Discov Today 20, 114-121.

815   Calvo-Alvarez, E., Stamatakis, K., Punzon, C., Alvarez-Velilla, R., Tejeria, A., Escudero-
816   Martinez, J.M., Perez-Pertejo, Y., Fresno, M., Balana-Fouce, R., Reguera, R.M., 2015b.
817   Infrared fluorescent imaging as a potent tool for in vitro, ex vivo and in vivo models of visceral
818   leishmaniasis. PLoS Negl Trop Dis 9, e0003666.

819   Capewell, P., Cren-Travaille, C., Marchesi, F., Johnston, P., Clucas, C., Benson, R.A.,
820   Gorman, T.A., Calvo-Alvarez, E., Crouzols, A., Jouvion, G., Jamonneau, V., Weir, W.,
821   Stevenson, M.L., O'Neill, K., Cooper, A., Swar, N.K., Bucheton, B., Ngoyi, D.M., Garside, P.,
822   Rotureau, B., MacLeod, A., 2016. The skin is a significant but overlooked anatomical
823   reservoir for vector-borne African trypanosomes. eLife 5.

824   Caridha, D., Parriot, S., Hudson, T.H., Lang, T., Ngundam, F., Leed, S., Sena, J., Harris, M.,
825   O'Neil, M., Sciotti, R., Read, L., Lecoeur, H., Hickman, M., Grogl, M., 2017. Use of Optical
826   Imaging Technology in the Validation of a New, Rapid, Cost-Effective Drug Screen as Part of
827   a Tiered In Vivo Screening Paradigm for Development of Drugs To Treat Cutaneous
828   Leishmaniasis. Antimicrob Agents Chemother 61.

829   Carneiro, M.B., Hohman, L.S., Egen, J.G., Peters, N.C., 2017. Use of two-photon microscopy
830   to study Leishmania major infection of the skin. Methods.

831   Claes, F., Vodnala, S.K., van Reet, N., Boucher, N., Lunden-Miguel, H., Baltz, T., Goddeeris,
832   B.M., Buscher, P., Rottenberg, M.E., 2009. Bioluminescent imaging of Trypanosoma brucei
833   shows preferential testis dissemination which may hamper drug efficacy in sleeping sickness.
834   PLoS Negl Trop Dis 3, e486.

835   Coles, J.A., Stewart-Hutchinson, P.J., Myburgh, E., Brewer, J.M., 2017. The mouse cortical
836   meninges are the site of immune responses to many different pathogens, and are accessible
837   to intravital imaging. Methods.

838   Coombes, J.L., Robey, E.A., 2010. Dynamic imaging of host-pathogen interactions in vivo.
839   Nat Rev Immunol 10, 353-364.

840   D'Archivio, S., Cosson, A., Medina, M., Lang, T., Minoprio, P., Goyard, S., 2013. Non-
841   invasive in vivo study of the Trypanosoma vivax infectious process consolidates the brain
842   commitment in late infections. PLoS Negl Trop Dis 7, e1976.

843   Dias Fde, A., Guerra, B., Vieira, L.R., Perdomo, H.D., Gandara, A.C., Amaral, R.J., Vollu,
844   R.E., Gomes, S.A., Lara, F.A., Sorgine, M.H., Medei, E., de Oliveira, P.L., Salmon, D., 2015.
845   Monitoring of the Parasite Load in the Digestive Tract of Rhodnius prolixus by Combined

846 qPCR Analysis and Imaging Techniques Provides New Insights into the Trypanosome Life
847 Cycle. PLoS Negl Trop Dis 9, e0004186.

848 Dias, J.C., 2015. Evolution of Chagas disease screening programs and control programs:
849 historical perspective. Glob Heart 10, 193-202.

850 Ema, M., Takahashi, S., Rossant, J., 2006. Deletion of the selection cassette, but not cis-
851 acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent
852 mesodermal progenitors. Blood 107, 111-117.

853 Forestier, C.L., 2013. Imaging host-Leishmania interactions: significance in visceral
854 leishmaniasis. Parasite Immunol 35, 256-266.

855 Franco, J.R., Cecchi, G., Priotto, G., Paone, M., Diarra, A., Grout, L., Mattioli, R.C., Argaw,
856 D., 2017. Monitoring the elimination of human African trypanosomiasis: Update to 2014.
857 PLoS Negl Trop Dis 11, e0005585.

858 Gonzalez, U., Pinart, M., Sinclair, D., Firooz, A., Enk, C., Velez, I.D., Esterhuizen, T.M.,
859 Tristan, M., Alvar, J., 2015. Vector and reservoir control for preventing leishmaniasis.
860 Cochrane Database Syst Rev, CD008736.

861 Huet, D., Blisnick, T., Perrot, S., Bastin, P., 2014. The GTPase IFT27 is involved in both
862 anterograde and retrograde intraflagellar transport. eLife 3, e02419.

863 Julkowska, D., Bastin, P., 2009. Tools for analyzing intraflagellar transport in trypanosomes.
864 Methods Cell Biol 93, 59-80.

865 Kapler, G.M., Zhang, K., Beverley, S.M., 1990. Nuclease mapping and DNA sequence
866 analysis of transcripts from the dihydrofolate reductase-thymidylate synthase (R) region of
867 Leishmania major. Nucleic Acids Res 18, 6399-6408.

868 Kohl, L., Sherwin, T., Gull, K., 1999. Assembly of the paraflagellar rod and the flagellum
869 attachment zone complex during the Trypanosoma brucei cell cycle. J Eukaryot Microbiol 46,
870 105-109.

871 Kooy, R.F., Hirumi, H., Moloo, S.K., Nantulya, V.M., Dukes, P., Van der Linden, P.M.,
872 Duijndam, W.A., Janse, C.J., Overdulve, J.P., 1989. Evidence for diploidy in metacyclic
873 forms of African trypanosomes. Proc Natl Acad Sci U S A 86, 5469-5472.

874 Lang, T., Lecoeur, H., Prina, E., 2009. Imaging Leishmania development in their host cells.
875 Trends Parasitol 25, 464-473.

876 Le Ray, D., Barry, J.D., Easton, C., Vickerman, K., 1977. First tsetse fly transmission of the
877 "AnTat" serodeme of Trypanosoma brucei. Ann Soc Belg Med Trop 57, 369-381.

878 Lewis, M.D., Francisco, A.F., Taylor, M.C., Kelly, J.M., 2015. A new experimental model for
879 assessing drug efficacy against Trypanosoma cruzi infection based on highly sensitive in
880 vivo imaging. J Biomol Screen 20, 36-43.

881 Lim, E., Modi, K., Christensen, A., Meganck, J., Oldfield, S., Zhang, N., 2011. Monitoring
882 tumor metastases and osteolytic lesions with bioluminescence and micro CT imaging. J Vis
883 Exp.

884   Lima, H.C., Bleyenberg, J.A., Titus, R.G., 1997. A simple method for quantifying Leishmania
885   in tissues of infected animals. Parasitol Today 13, 80-82.

886   Liu, H., Patel, M.R., Prescher, J.A., Patsialou, A., Qian, D., Lin, J., Wen, S., Chang, Y.F.,
887   Bachmann, M.H., Shimono, Y., Dalerba, P., Adorno, M., Lobo, N., Bueno, J., Dirbas, F.M.,
888   Goswami, S., Somlo, G., Condeelis, J., Contag, C.H., Gambhir, S.S., Clarke, M.F., 2010.
889   Cancer stem cells from human breast tumors are involved in spontaneous metastases in
890   orthotopic mouse models. Proc Natl Acad Sci U S A 107, 18115-18120.

891   MacGregor, P., Rojas, F., Dean, S., Matthews, K.R., 2013. Stable transformation of
892   pleomorphic bloodstream form Trypanosoma brucei. Mol Biochem Parasitol 190, 60-62.

893   MacLean, L., Myburgh, E., Rodgers, J., Price, H.P., 2013. Imaging African trypanosomes.
894   Parasite Immunol 35, 283-294.

895   MacLeod, E.T., Maudlin, I., Darby, A.C., Welburn, S.C., 2007. Antioxidants promote
896   establishment of trypanosome infections in tsetse. Parasitology 134, 827-831.

897   Matlashewski, G., Arana, B., Kroeger, A., Be-Nazir, A., Mondal, D., Nabi, S.G., Banjara,
898   M.R., Das, M.L., Marasini, B., Das, P., Medley, G., Satoskar, A., Nakhasi, H., Argaw, D.,
899   Reeder, J., Olliaro, P., 2014. Research priorities for elimination of visceral leishmaniasis.
900   Lancet Glob Health 2, e683-684.

901   McLatchie, A.P., Burrell-Saward, H., Myburgh, E., Lewis, M.D., Ward, T.H., Mottram, J.C.,
902   Croft, S.L., Kelly, J.M., Taylor, M.C., 2013. Highly sensitive in vivo imaging of Trypanosoma
903   brucei expressing "red-shifted" luciferase. PLoS Negl Trop Dis 7, e2571.

904   Melo, G.D., Goyard, S., Fiette, L., Boissonnas, A., Combadiere, C., Machado, G.F., Minoprio,
905   P., Lang, T., 2017. Unveiling Cerebral Leishmaniasis: parasites and brain inflammation in
906   Leishmania donovani infected mice. Sci Rep 7, 8454.

907   Mezzanotte, L., Blankevoort, V., Lowik, C.W., Kaijzel, E.L., 2014. A novel luciferase fusion
908   protein for highly sensitive optical imaging: from single-cell analysis to in vivo whole-body
909   bioluminescence imaging. Analytical and bioanalytical chemistry 406, 5727-5734.

910   Myburgh, E., Coles, J.A., Ritchie, R., Kennedy, P.G., McLatchie, A.P., Rodgers, J., Taylor,
911   M.C., Barrett, M.P., Brewer, J.M., Mottram, J.C., 2013. In vivo imaging of trypanosome-brain
912   interactions and development of a rapid screening test for drugs against CNS stage
913   trypanosomiasis. PLoS Negl Trop Dis 7, e2384.

914   Ooi, C.P., Rotureau, B., Gribaldo, S., Georgikou, C., Julkowska, D., Blisnick, T., Perrot, S.,
915   Subota, I., Bastin, P., 2015. The Flagellar Arginine Kinase in Trypanosoma brucei Is
916   Important for Infection in Tsetse Flies. PLoS ONE 10, e0133676.

917   Osorio, Y., Travi, B.L., Renslo, A.R., Peniche, A.G., Melby, P.C., 2011. Identification of small
918   molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant
919   model system. PLoS Negl Trop Dis 5, e962.

920 Patel, M.R., Chang, Y.F., Chen, I.Y., Bachmann, M.H., Yan, X., Contag, C.H., Gambhir, S.S.,
921 2010. Longitudinal, noninvasive imaging of T-cell effector function and proliferation in living
922 subjects. Cancer Res 70, 10141-10149.

923 Peniche, A.G., Osorio, Y., Renslo, A.R., Frantz, D.E., Melby, P.C., Travi, B.L., 2014.
924 Development of an ex vivo lymph node explant model for identification of novel molecules
925 active against Leishmania major. Antimicrob Agents Chemother 58, 78-87.

926 Perez-Molina, J.A., Molina, I., 2017. Chagas disease. Lancet.

927 Reimao, J.Q., Oliveira, J.C., Trinconi, C.T., Cotrim, P.C., Coelho, A.C., Uliana, S.R., 2015.
928 Generation of luciferase-expressing Leishmania infantum chagasi and assessment of
929 miltefosine efficacy in infected hamsters through bioimaging. PLoS Negl Trop Dis 9,
930 e0003556.

931 Reithinger, R., Dujardin, J.C., Louzir, H., Pirmez, C., Alexander, B., Brooker, S., 2007.
932 Cutaneous leishmaniasis. Lancet Infect Dis 7, 581-596.

933 Rotureau, B., Ooi, C.P., Huet, D., Perrot, S., Bastin, P., 2014. Forward motility is essential for
934 trypanosome infection in the tsetse fly. Cell Microbiol 16, 425-433.

935 Rotureau, B., Subota, I., Bastin, P., 2011. Molecular bases of cytoskeleton plasticity during
936 the Trypanosoma brucei parasite cycle. Cell Microbiol 13, 705-716.

937 Rotureau, B., Subota, I., Buisson, J., Bastin, P., 2012. A new asymmetric division contributes
938 to the continuous production of infective trypanosomes in the tsetse fly. Development 139,
939 1842-1850.

940 Rotureau, B., Van Den Abbeele, J., 2013. Through the dark continent: African trypanosome
941 development in the tsetse fly. Frontiers in cellular and infection microbiology 3, 53.

942 Rouault, E., Lecoeur, H., Meriem, A.B., Minoprio, P., Goyard, S., Lang, T., 2017. Imaging
943 visceral leishmaniasis in real time with golden hamster model: Monitoring the parasite burden
944 and hamster transcripts to further characterize the immunological responses of the host.
945 Parasitol Int 66, 933-939.

946 Schuster, S., Kruger, T., Subota, I., Thusek, S., Rotureau, B., Beilhack, A., Engstler, M.,
947 2017. Developmental adaptations of trypanosome motility to the tsetse fly host environments
948 unravel a multifaceted in vivo microswimmer system. eLife 6.

949 Spath, G.F., Epstein, L., Leader, B., Singer, S.M., Avila, H.A., Turco, S.J., Beverley, S.M.,
950 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the
951 protozoan parasite Leishmania major. Proc Natl Acad Sci U S A 97, 9258-9263.

952 Stanaway, J.D., Roth, G., 2015. The burden of Chagas disease: estimates and challenges.
953 Glob Heart 10, 139-144.

954 Taheri, T., Saberi Nik, H., Seyed, N., Doustdari, F., Etemadzadeh, M.H., Torkashvand, F.,
955 Rafati, S., 2015. Generation of stable L. major(+EGFP-LUC) and simultaneous comparison
956 between EGFP and luciferase sensitivity. Exp Parasitol 150, 44-55.

957 Takai, A., Nakano, M., Saito, K., Haruno, R., Watanabe, T.M., Ohyanagi, T., Jin, T., Okada,

958 Y., Nagai, T., 2015. Expanded palette of Nano-lanterns for real-time multicolor luminescence

959 imaging. Proc Natl Acad Sci U S A 112, 4352-4356.

960 Trindade, S., Rijo-Ferreira, F., Carvalho, T., Pinto-Neves, D., Guegan, F., Aresta-Branco, F.,

961 Bento, F., Young, S.A., Pinto, A., Van Den Abbeele, J., Ribeiro, R.M., Dias, S., Smith, T.K.,

962 Figueiredo, L.M., 2016. Trypanosoma brucei Parasites Occupy and Functionally Adapt to the

963 Adipose Tissue in Mice. Cell Host Microbe.

964 Van Reet, N., Van de Vyver, H., Pyana, P.P., Van der Linden, A.M., Buscher, P., 2014. A

965 panel of Trypanosoma brucei strains tagged with blue and red-shifted luciferases for

966 bioluminescent imaging in murine infection models. PLoS Negl Trop Dis 8, e3054.

967 Willmann, J.K., van Bruggen, N., Dinkelborg, L.M., Gambhir, S.S., 2008. Molecular imaging

968 in drug development. Nat Rev Drug Discov 7, 591-607.

969 Xong, H.V., Vanhamme, L., Chamekh, M., Chimfwembe, C.E., Van Den Abbeele, J., Pays,

970 A., Van Meirvenne, N., Hamers, R., De Baetselier, P., Pays, E., 1998. A VSG expression

971 site-associated gene confers resistance to human serum in Trypanosoma rhodesiense. Cell

972 95, 839-846.

973

WT *L. major*

RED *L. major*

A — DNA

B — DNA / Red fluorescence

C — Plot P03, ungated — Count vs Fluorescence

D — Plot P03, ungated — Count vs Fluorescence

E — No. of parasites (x10^6) vs Time (days)
- WT Lm
- RED Lm
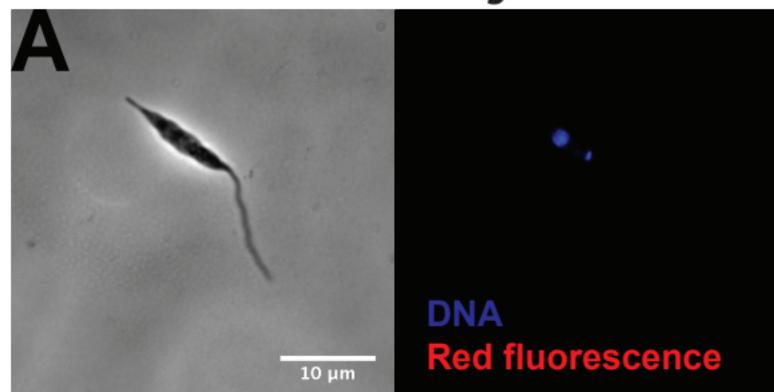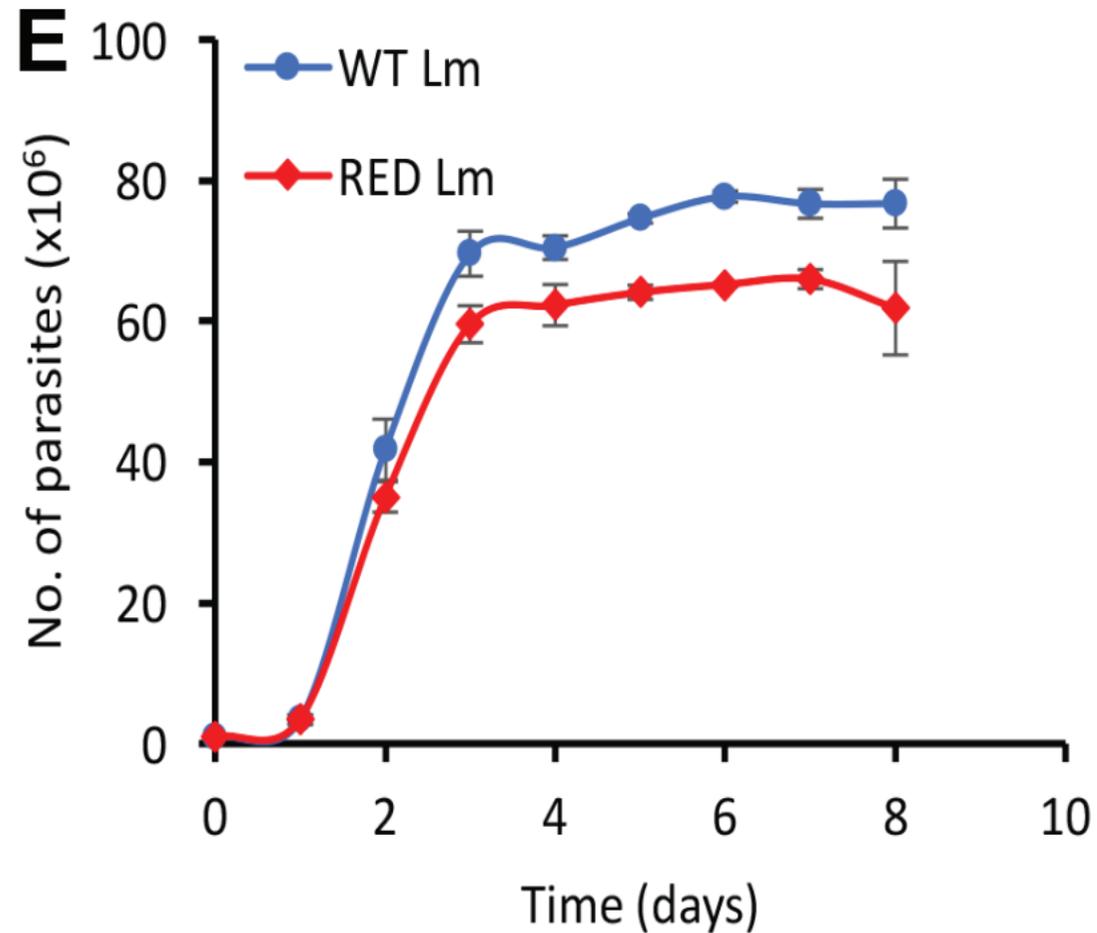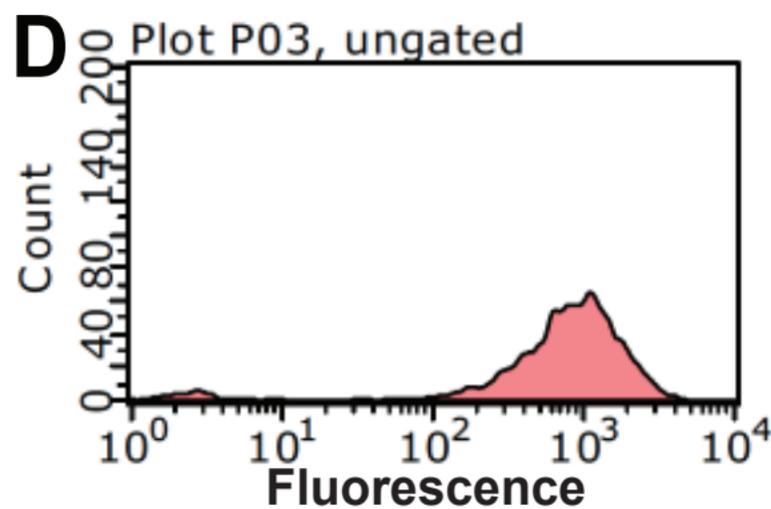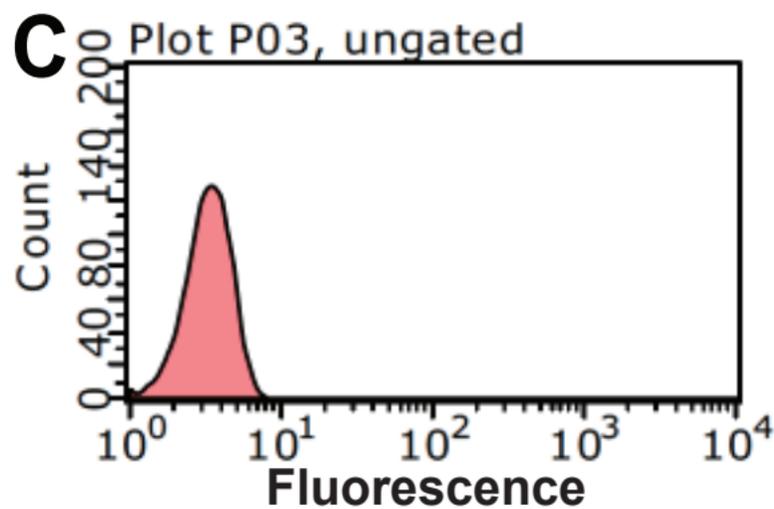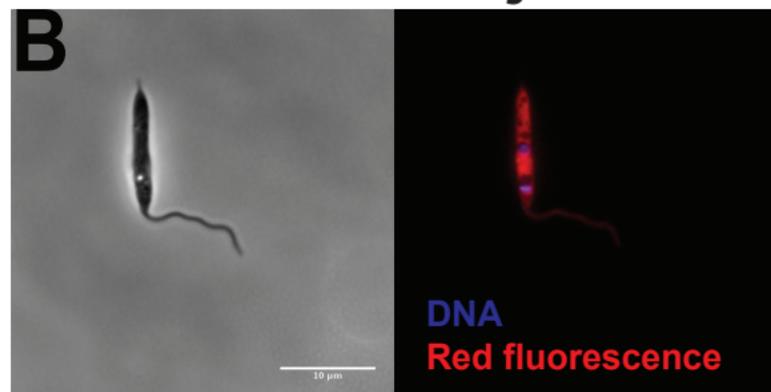
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Fluorescence
(Radiance in p/s/cm²/sr)
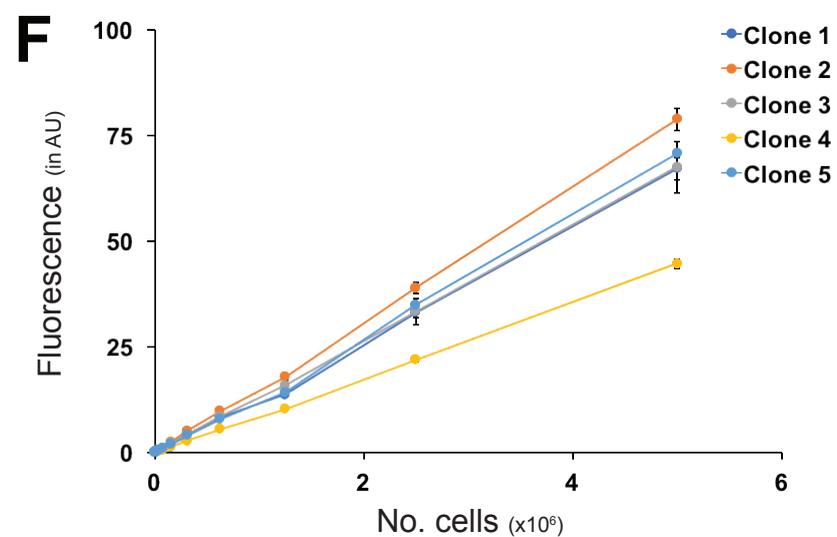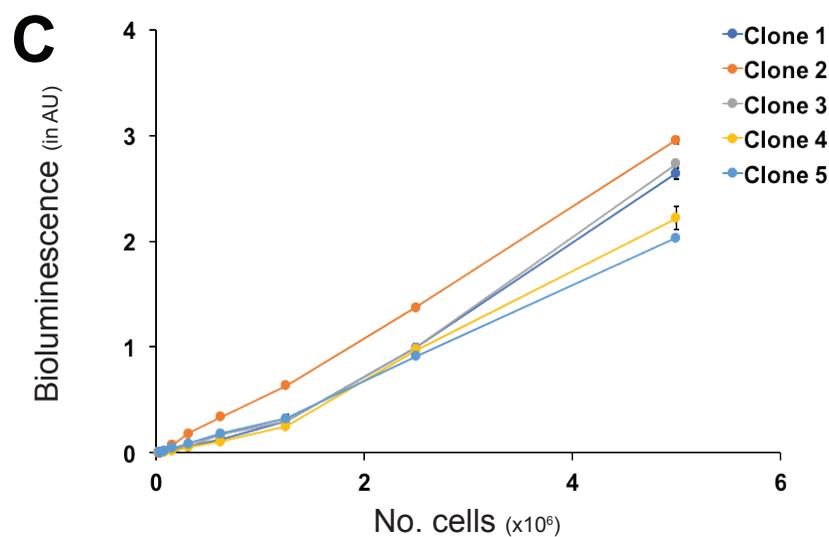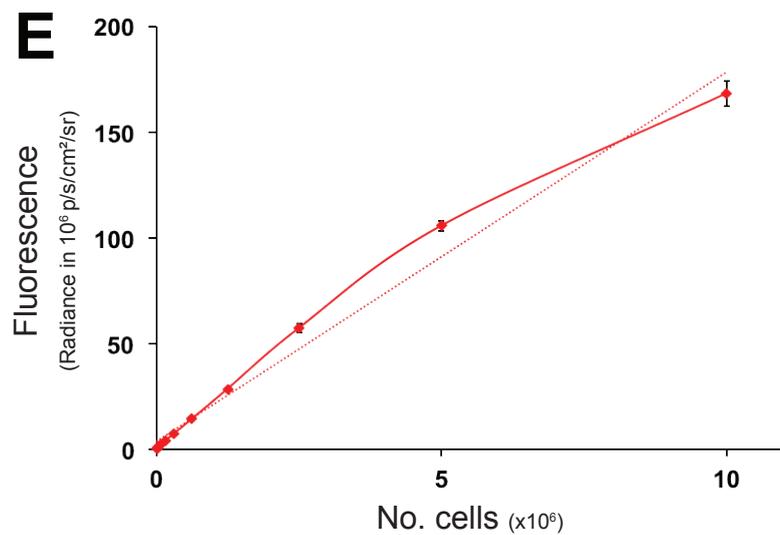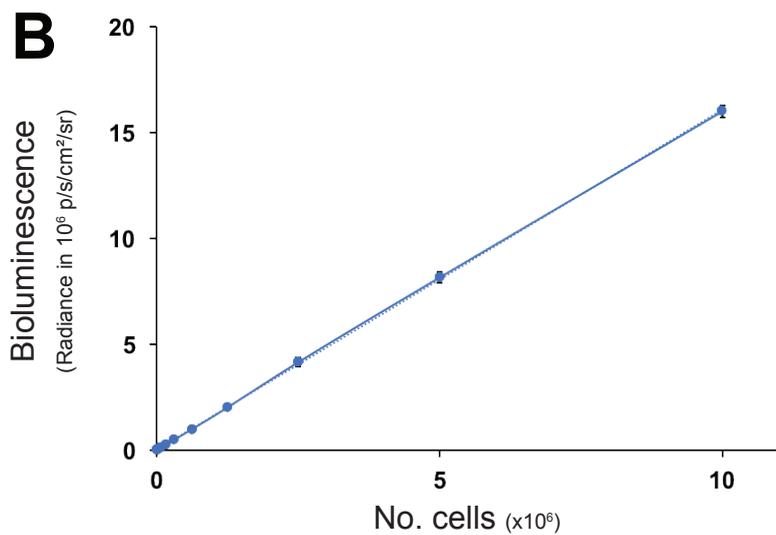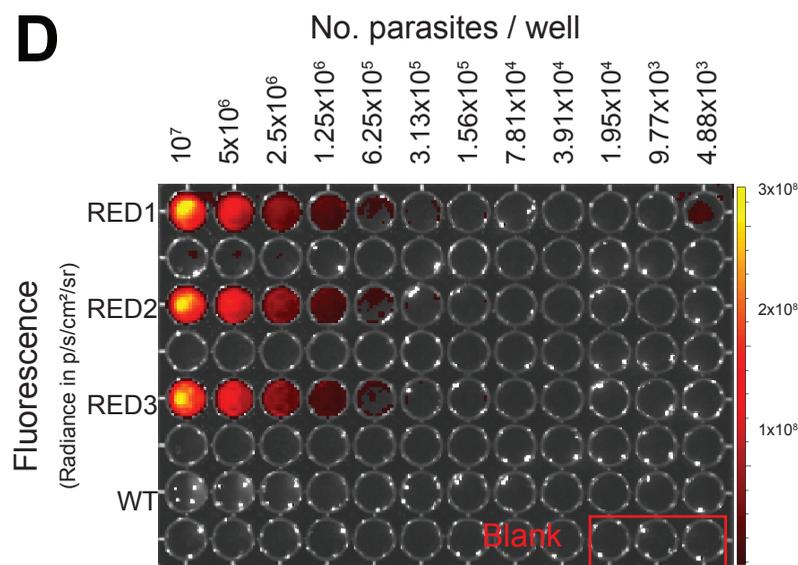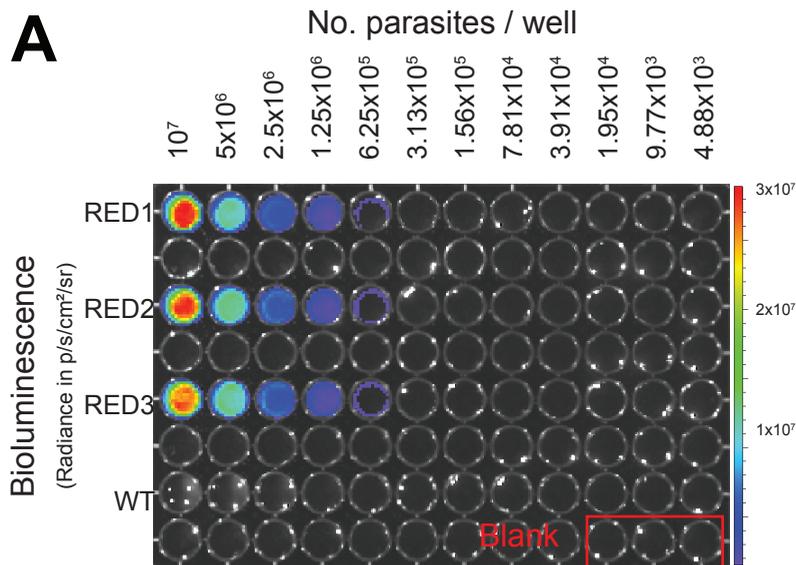
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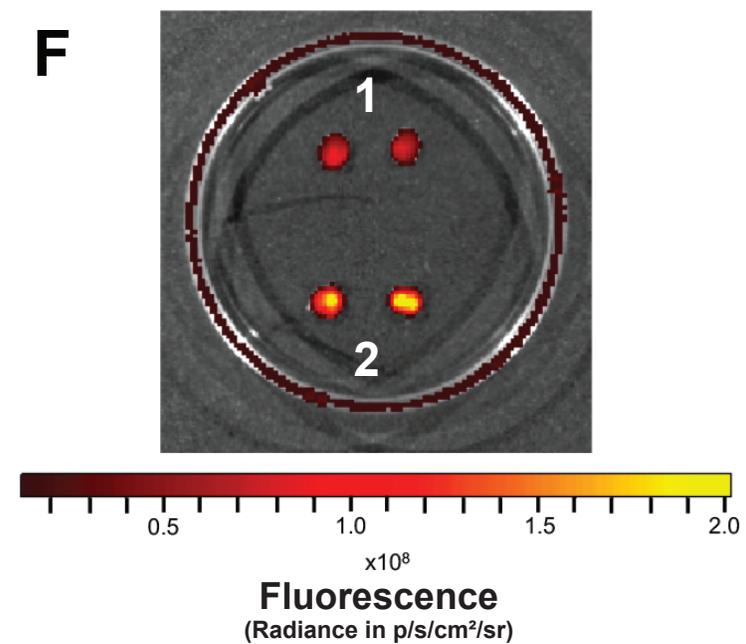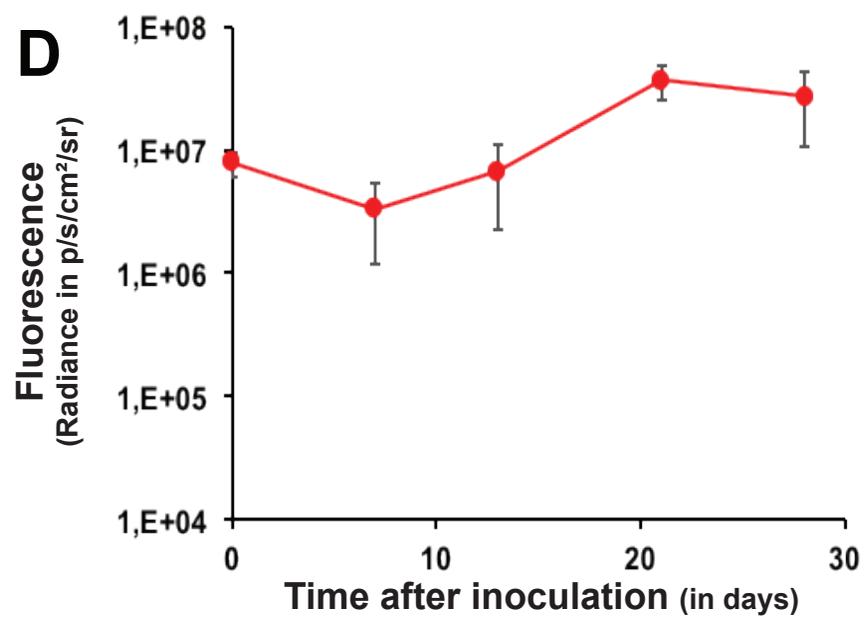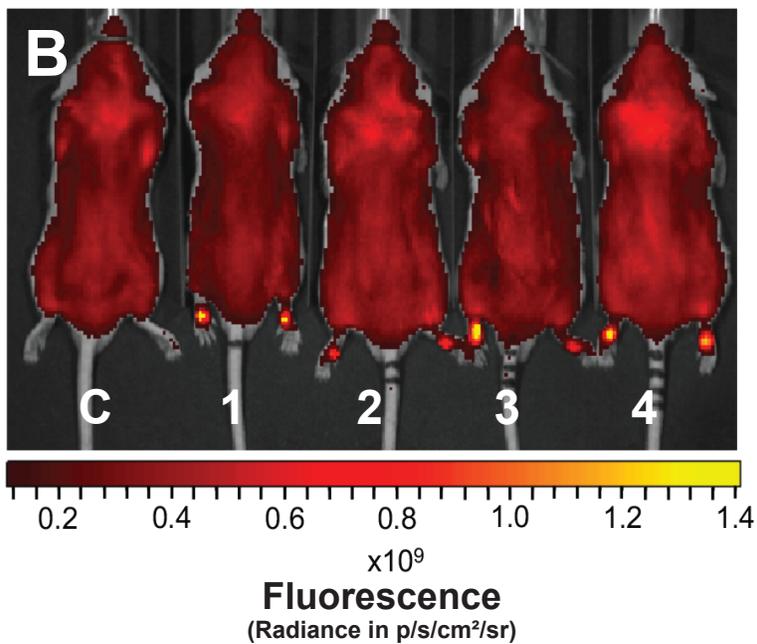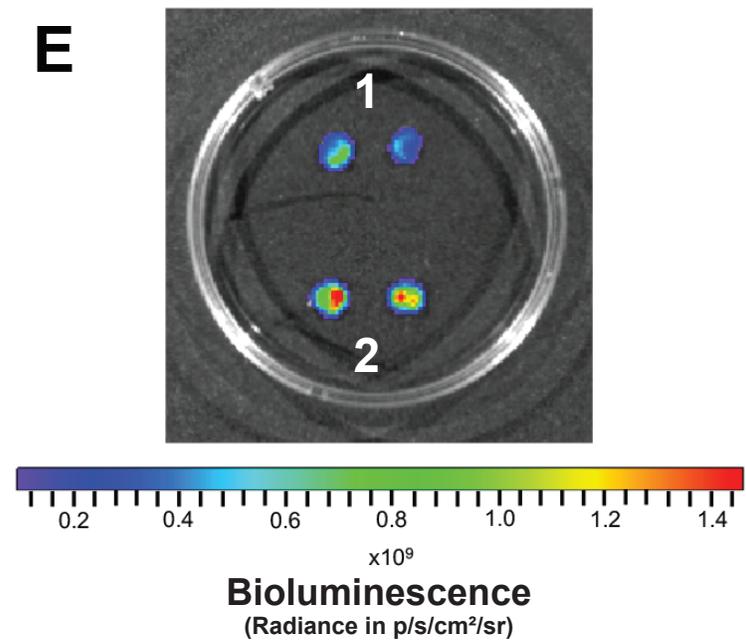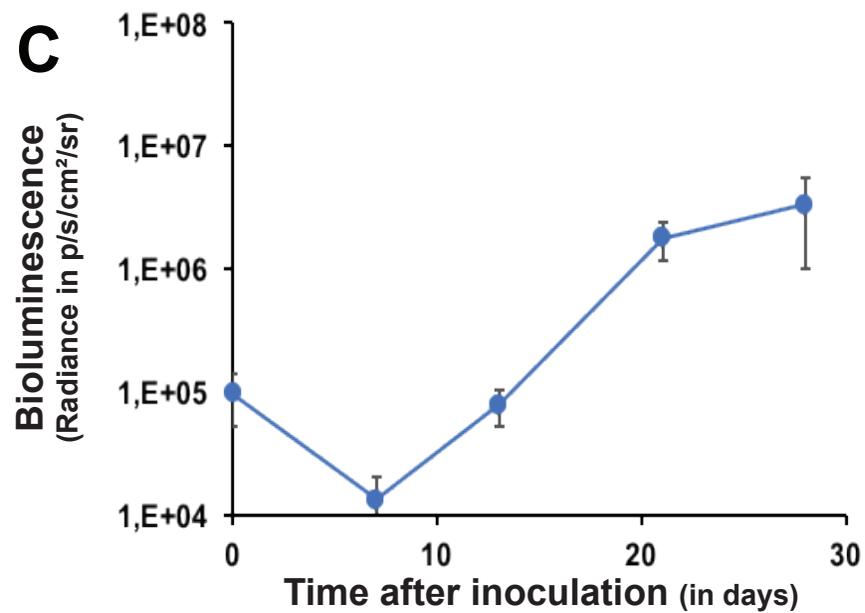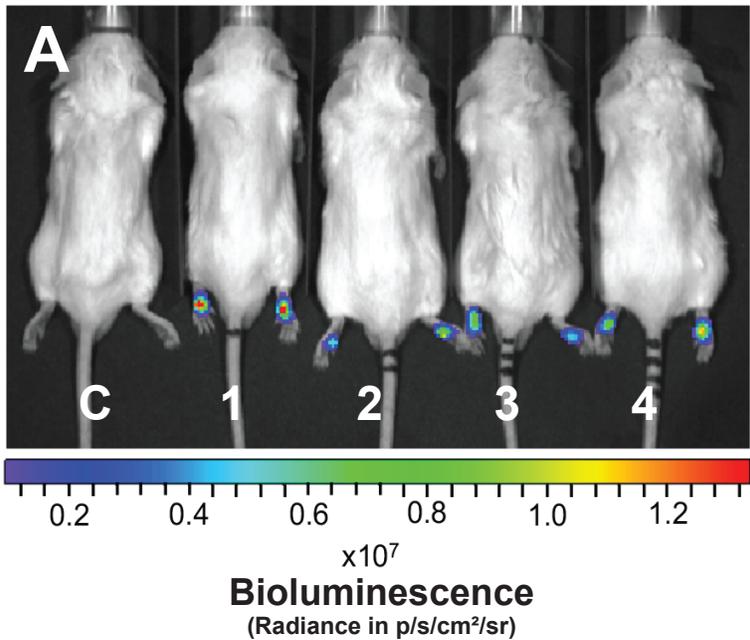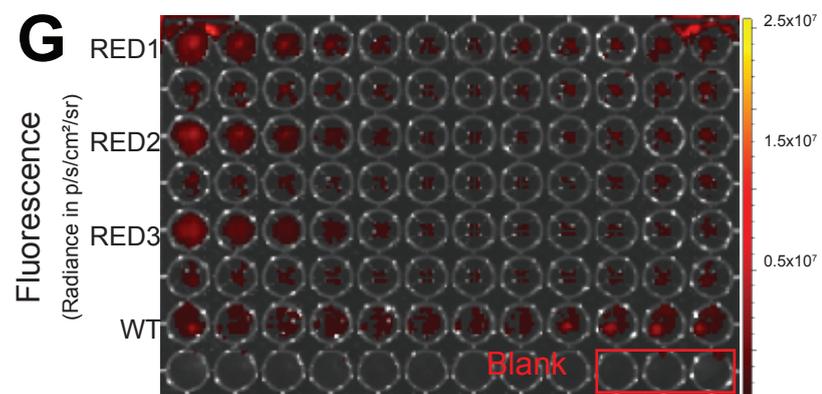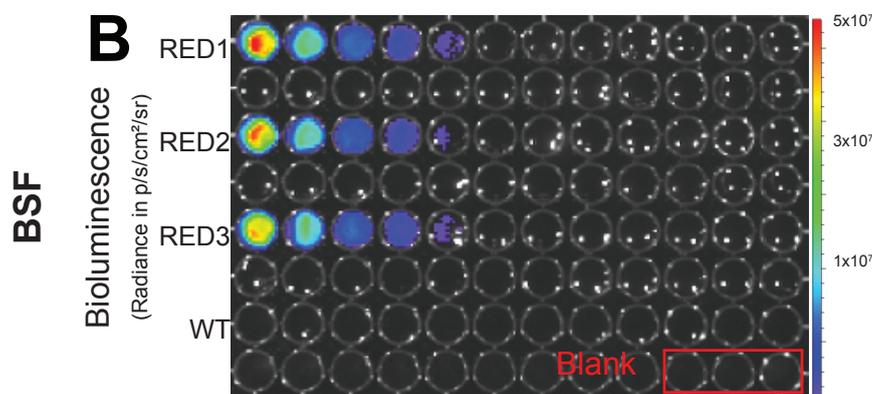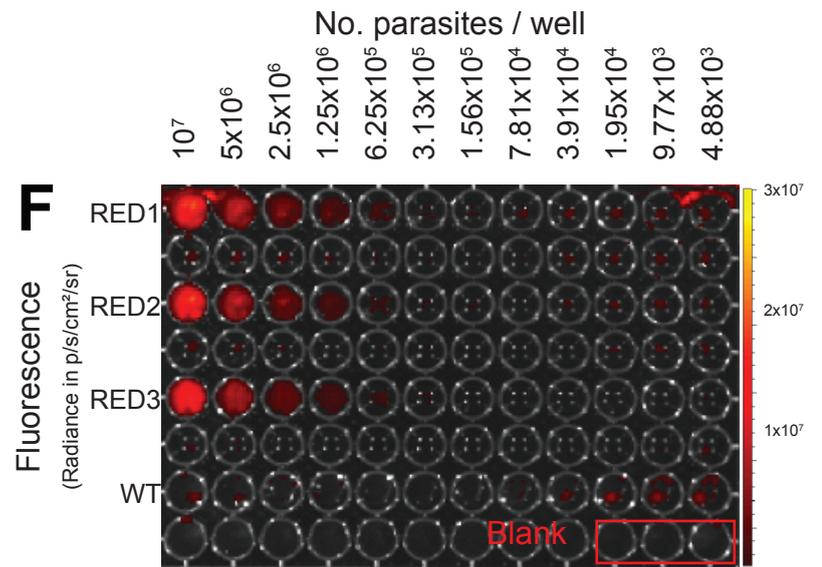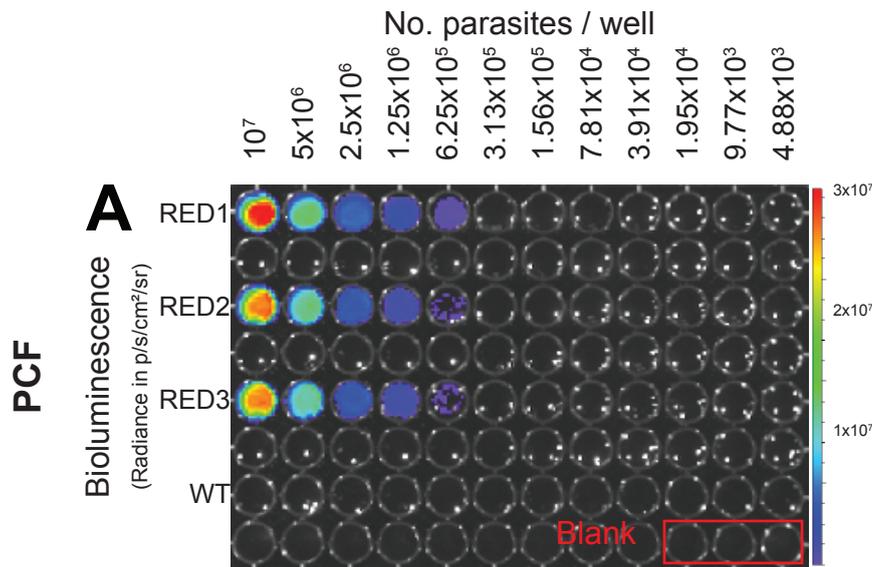
WT *T. b. brucei*   **RED *T. b. brucei***

PCF

A DNA / Red fluorescence — 10 µm

B DNA / Red fluorescence — 10 µm

C Plot P03, ungated

D Plot P03, ungated

E DNA / Red fluorescence — 10 µm

F DNA / Red fluorescence — 10 µm

BSF

G Plot P03, ungated

H Plot P03, ungated

I
- WT PCFs
- RED PCFs

No. of parasites (x10^6) vs Time (days)

J
- WT BSFs
- RED BSFs

No. of parasites (x10^6) vs Time (days)
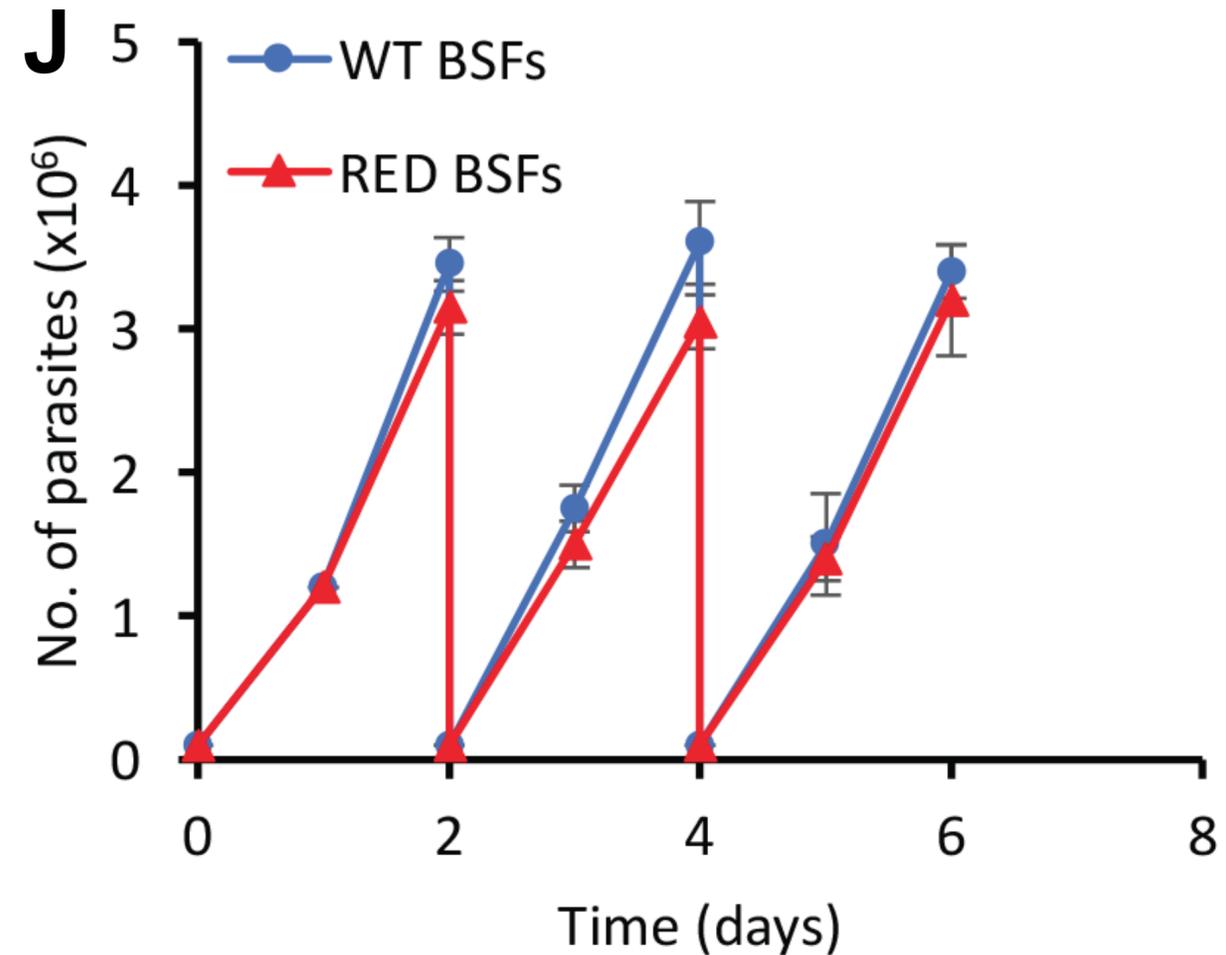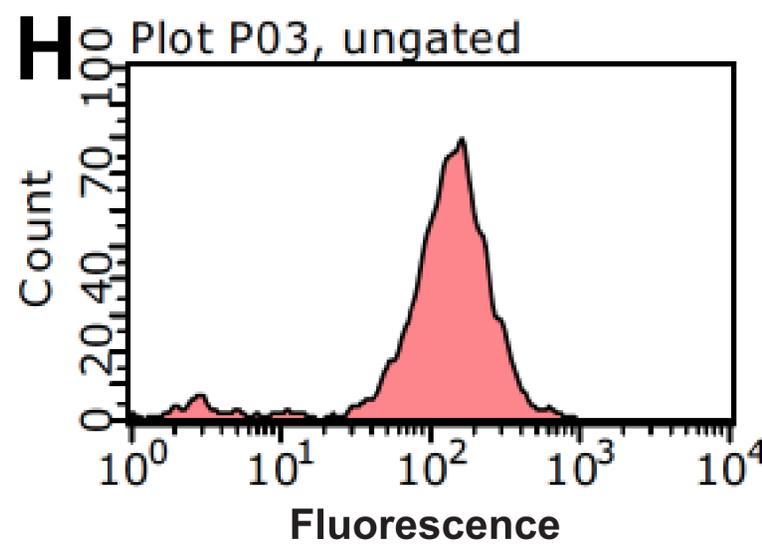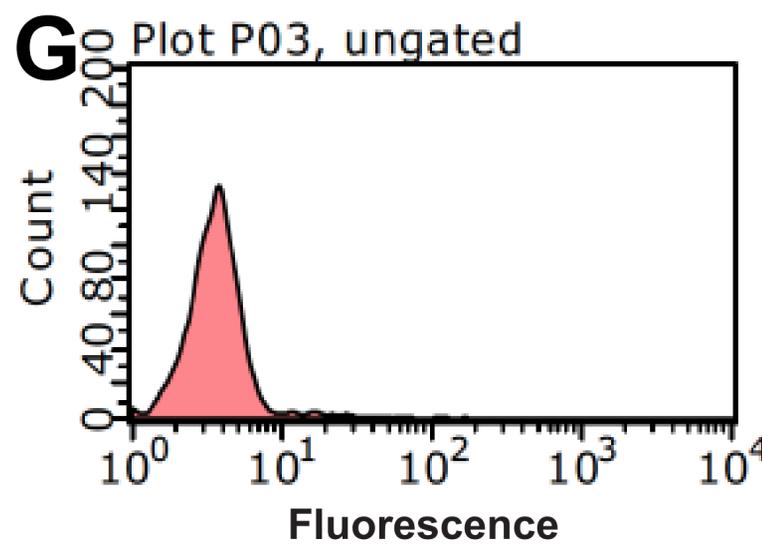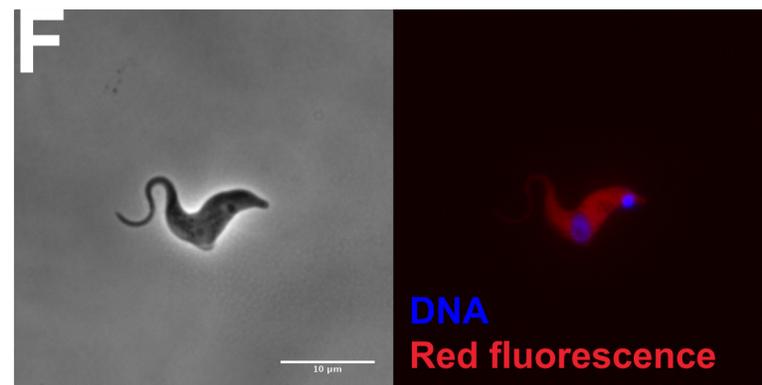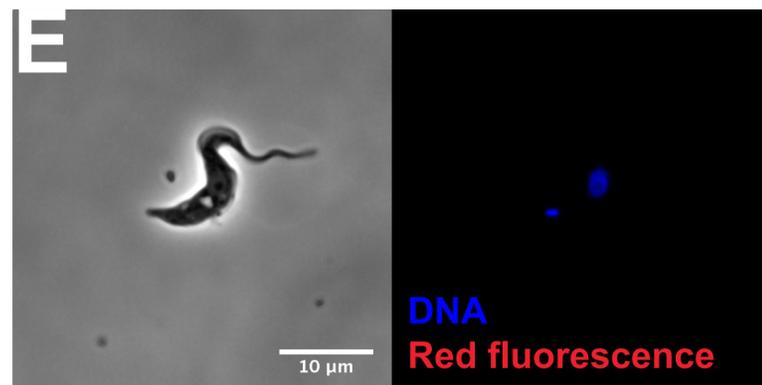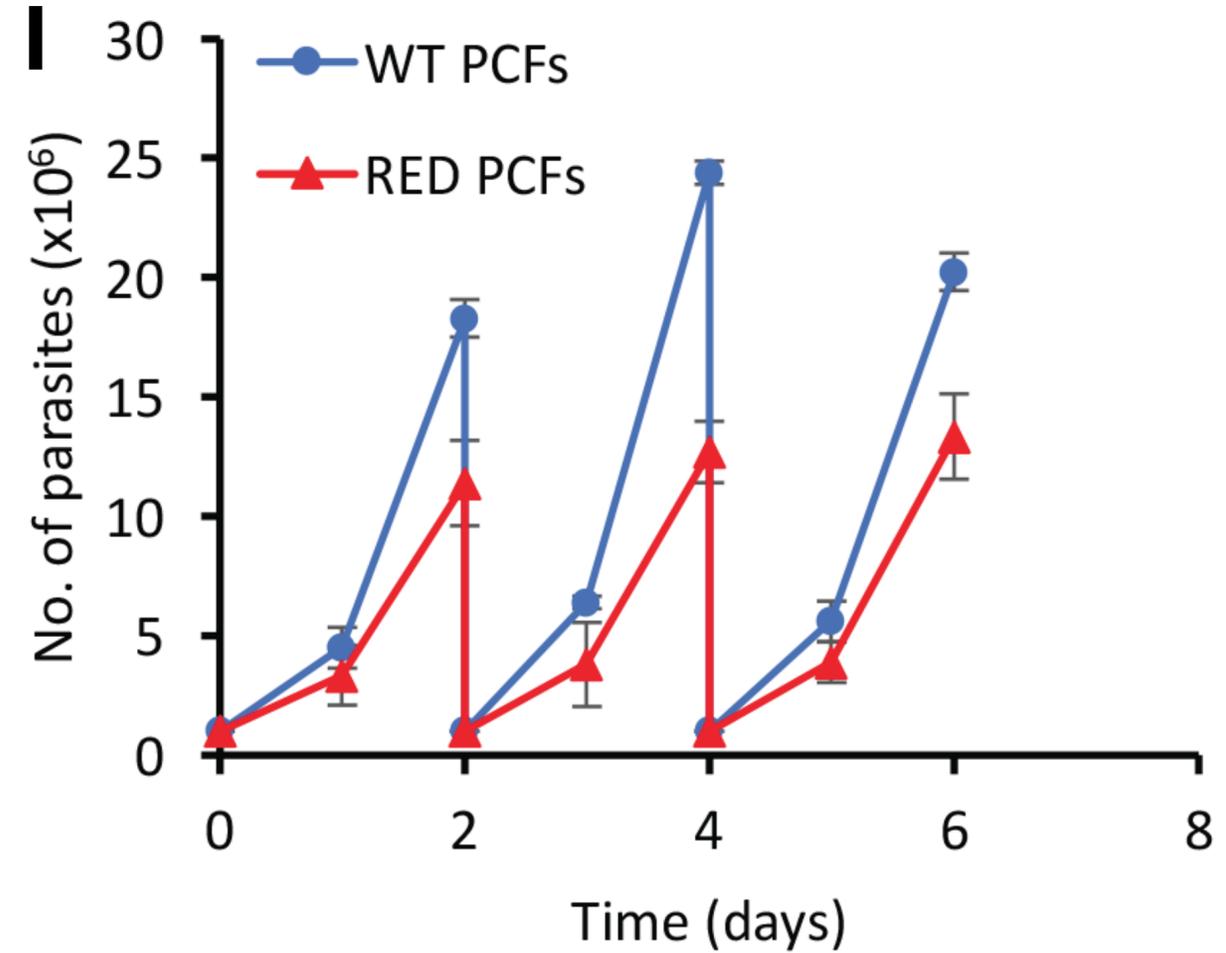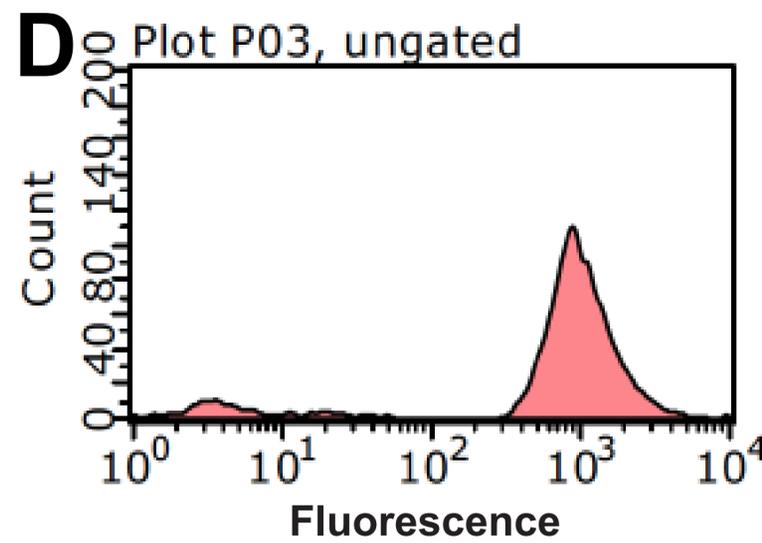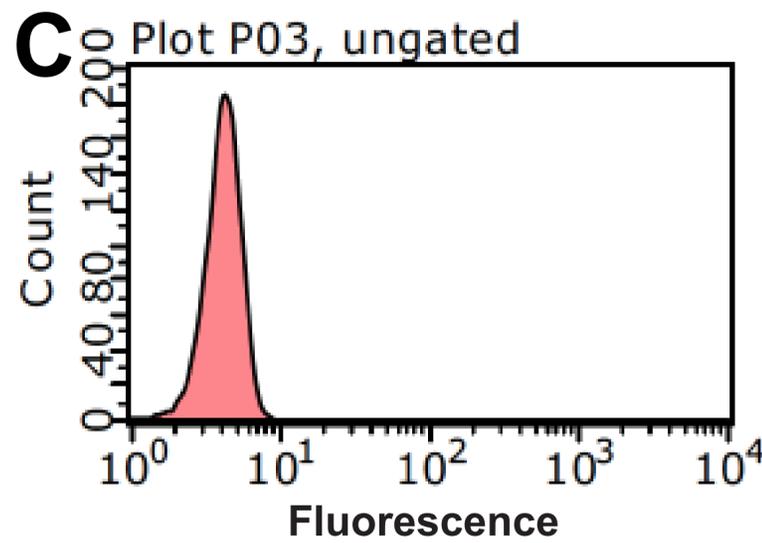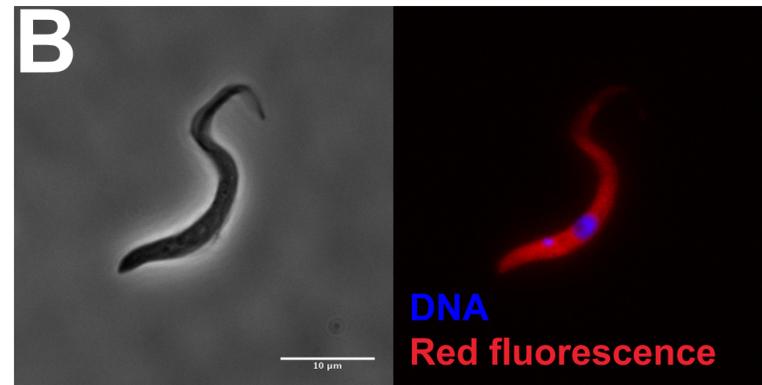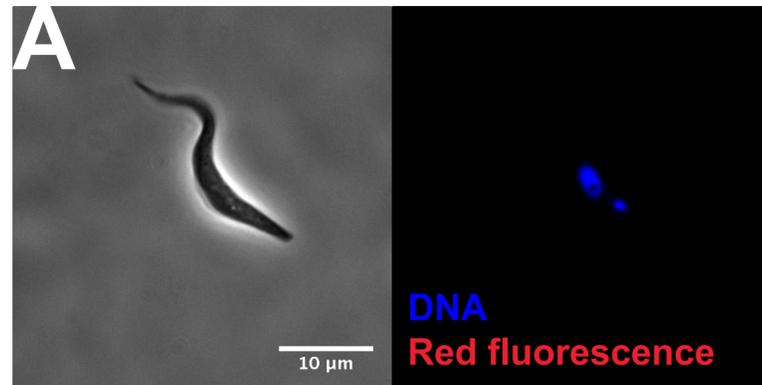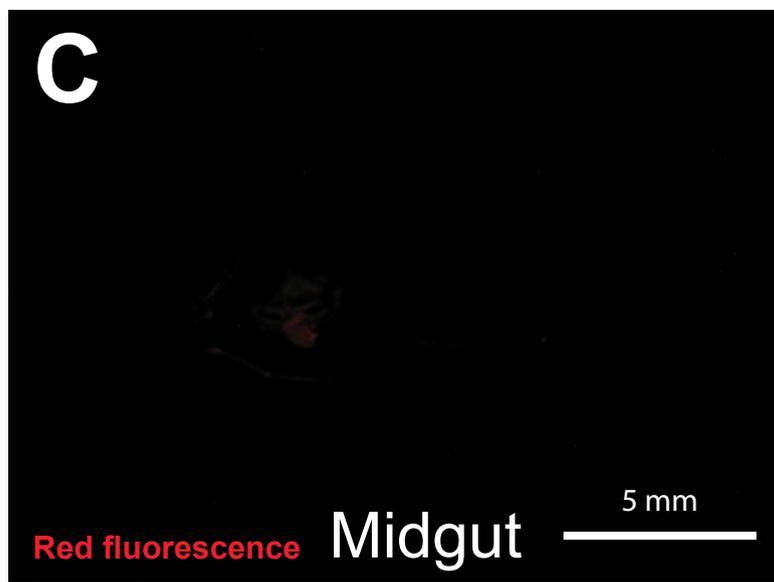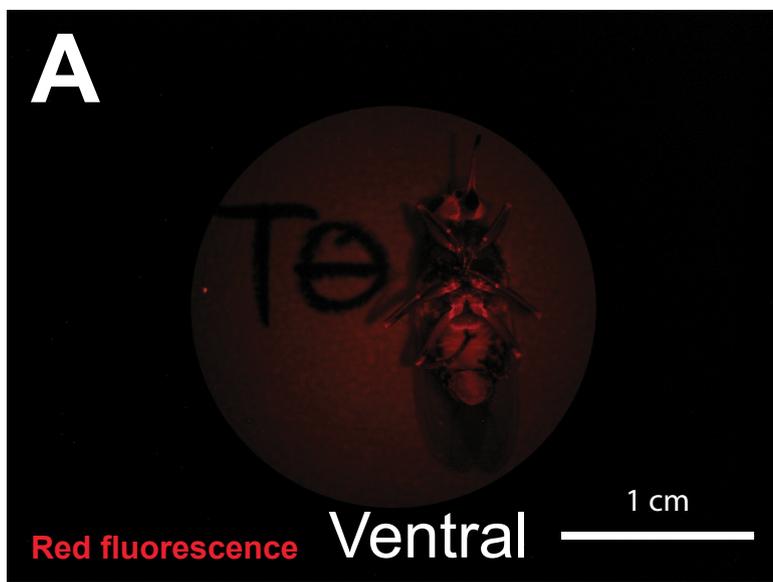
Bioluminescence
(Radiance in p/s/cm²/sr)

Fluorescence
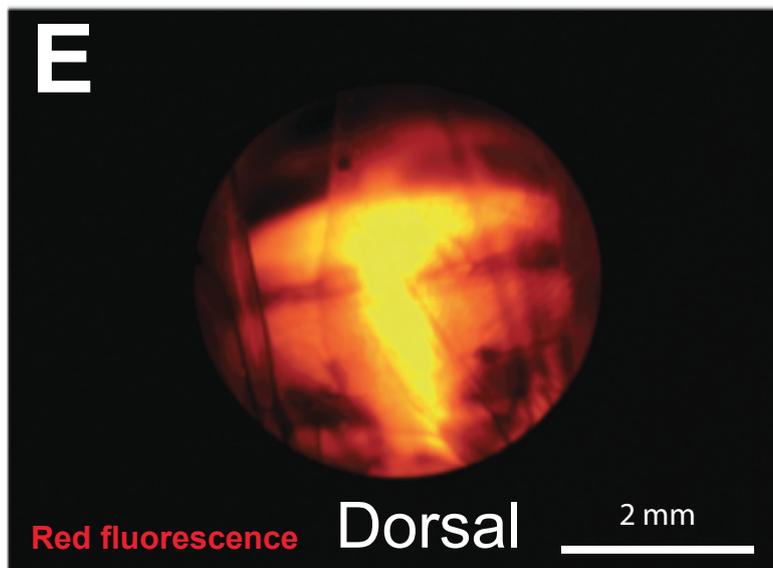(Radiance in p/s/cm²/sr)

In vivo imager

In vitro plate reader

A − RED parasites · Red fluorescence · Ventral · 1 cm

C Red fluorescence · Midgut · 5 mm

B + RED parasites · Red fluorescence · Ventral · 1 cm

D Red fluorescence · Midgut · 5 mm

E Red fluorescence · Dorsal · 2 mm

F Red fluorescence · Midgut · 1 mm

G

Infection rates (in %)

■ % MG+ ■ % SG+

WT (125) · RED (122) — BSF
WT (231) · RED (128) — PCF

Parasite strains

A — 8 days / 15 days / 22 days

Bioluminescence (Radiance in p/s/cm²/sr)

B — DNA / Trypanosomes (TdTomato) / 10 µm

C

Ventral BLI — Dorsal BLI — Blood

Bioluminescence (Radiance in p/s/cm²/sr)

Parasitemia (in parasites/ml)

Time after infective bite (in days)

D — Coronal / Sagittal / Transaxial / 3D

Bioluminescence

photons/sec

A

DNA
TY1

B

DNA
TY1

C

	L	*T. brucei*						*L. major*						L
		WT			RED			WT			RED			
		10^6	10^5	10^4	10^6	10^5	10^4	10^6	10^5	10^4	10^6	10^5	10^4	

TY1

PFR

250
150
100
75

D

DNA
TY1

A

PpyRE9h (548 aa) — TY1 (10 aa) — TdTomato (476 aa)

B

```
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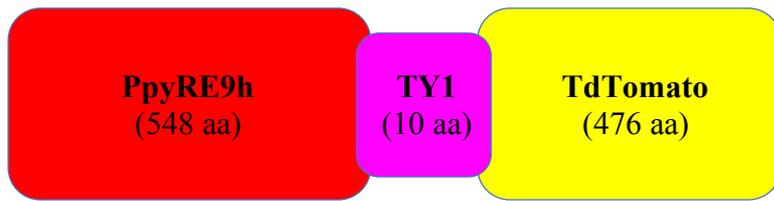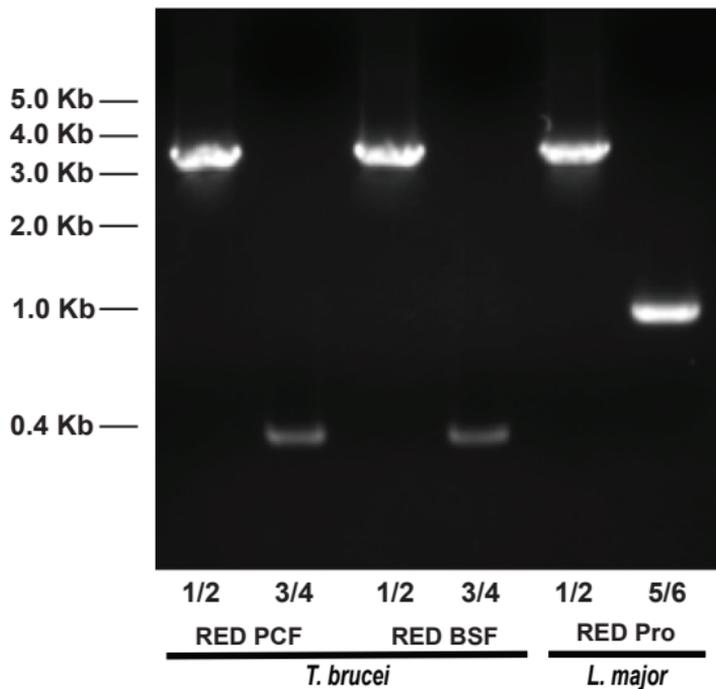
A

5.0 Kb —
4.0 Kb —
3.0 Kb —
2.0 Kb —

1.0 Kb —

0.4 Kb —

1/2	3/4	1/2	3/4	1/2	5/6
RED PCF		RED BSF		RED Pro	
T. brucei				*L. major*	

B

RED *T. brucei*

rDNA promoter

RRNA — PARP SL — *PpyRE9H-TY1-tdTomato* — PARP — *BSD* — Actin — RRNA

1 →   3 →
← 2   ← 4

RED *L. major*

18S RRNA — utr1 — *PpyRE9H-TY1-tdTomato* — utr2 — *HYG* — utr3 — 18S RRNA

1 →   5 →
← 2   ← 6

A

DNA
RED *Leishmania*

DNA
RED *Leishmania*

B

Endothelial cells (EGFP)
Trypanosomes (TdTomato)