Chapter 48

In Vivo Bioluminescence Imaging to Assess Compound Efficacy Against *Trypanosoma brucei*

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Abstract

Traditional animal models for human African trypanosomiasis rely on detecting *Trypanosoma brucei brucei* parasitemia in the blood. Testing the efficacy of new compounds in these models is cumbersome because it may take several months after treatment before surviving parasites become detectable in the blood. To expedite compound screening, we have used a *Trypanosoma brucei brucei* GVR35 strain expressing red-shifted firefly luciferase to monitor parasite distribution in infected mice through noninvasive whole-body bioluminescence imaging. This protocol describes the infection and in vivo bioluminescence imaging of mice to assess compound efficacy against *T. brucei* during the two characteristic stages of disease, the hemolymphatic phase (stage 1) and the encephalitic or central nervous system phase (stage 2).

Key words Firefly luciferase, *Trypanosoma brucei brucei*, In vivo imaging, Bioluminescence

1 Introduction

Human African trypanosomiasis (HAT), also called sleeping sickness, occurs in two clinical stages: a hemolymphatic phase (stage 1), where parasites are detected in blood and lymph, and an encephalitic phase (stage 2) involving the central nervous system (CNS). Established mouse models for HAT rely on detection of blood parasites, usually of monomorphic *T. brucei* strain Lister 427 during the first few days of infection (to mimic stage 1 disease), and of pleomorphic *T. brucei* strain GVR35 after 21 days of infections (to mimic stage 2 disease) [1, 2]. This involves sampling of blood for parasite detection by light microscopy and does not allow real-time detection of parasites that are extravascular and within tissues such as the spleen, lymph nodes, adipose tissue, and brain.

In vivo imaging is highly sensitive, noninvasive, and quantifiable, and has become an indispensable and valuable tool for the monitoring of disease progression in live animals. This technology has been applied to investigate infection dynamics and to screen

drugs against *Plasmodium* spp. [3–5], *Mycobacterium tuberculosis* [6], *Leishmania* spp. [7, 8], *Trypanosoma cruzi* [9], and *Trypanosoma brucei* [10, 11]. To make use of this technology for the screening of novel trypanocidal compounds, bioluminescent *Trypanosoma brucei brucei* cell lines were generated [12, 13] and an imaging method that is highly sensitive, reproducible and expedites the screening process was developed [14]. The optimized bioluminescence imaging model using *T. b. brucei* GVR35 expressing red-shifted firefly luciferase has been valuable for the identification of novel compounds against stage 2 HAT [15]. This bioluminescence GVR35 model can also be utilized to screen for in vivo activity during stage 1 disease. Infection of mice with pleomorphic *T. brucei* strains is characterized by waves of parasitemia that can be difficult to detect in the blood. However, sensitive bioluminescence imaging tracks live parasites over the whole body making it possible to assess compound activity even with fluctuating and low blood parasitemia. Using one parasite strain and method to screen for both stage 1 and 2 efficacy saves limited time and resources routinely spent to assess compounds in multiple parasite strains and infection models.

In this protocol, we describe the generation and testing of bioluminescent *T. b. brucei* GVR35 stabilates to be used for reproducible infections of mice. We describe the imaging model (Fig. 1) for screening of compounds against pleomorphic *T. b. brucei* GVR35 during stage 1 (day 7 postinfection) or stage 2 (day 21 postinfection) trypanosomiasis. We provide details on infection of donor and experimental mice, bioluminescence imaging of mice (using the IVIS imaging system from PerkinElmer), determination of blood parasitemia, treatment of mice with compounds, blood sampling for pharmacokinetic analysis and end-point imaging and harvesting of organs to allow parasite quantification by PCR (*see* **Note 1**).

2   Materials

2.1 Generation and Testing of Bioluminescent T. brucei *Stabilates*

2.1.1 Culturing of T. b. brucei *GVR35 Bloodstream form Trypanosomes*

1. *Trypanosoma brucei brucei* GVR35-VSL2 [12] (*see* **Note 2**).

2. IMDM (Iscove's Modified Dulbecco's Medium, Gibco) supplemented with 20% heat-inactivated fetal calf serum, 20% Serum Plus, 0.75 mM hypoxanthine in 0.1 N NaOH, 4.1 mM glucose, 0.12 mM thymidine, 1.5 mM sodium pyruvate, 0.037 mM bathocuproine disulfonic acid, 0.2 mM β-mercaptoethanol, 1.1 mM L-cysteine, 0.38 mM adenosine, 0.38 mM guanosine, 0.83 g/L methylcellulose, 0.04 mM kanamycin, 75 units/mL penicillin, and 0.075 mg/mL streptomycin (all Sigma-Aldrich), pH 7.4.

3. 25 cm^3 vented flasks.
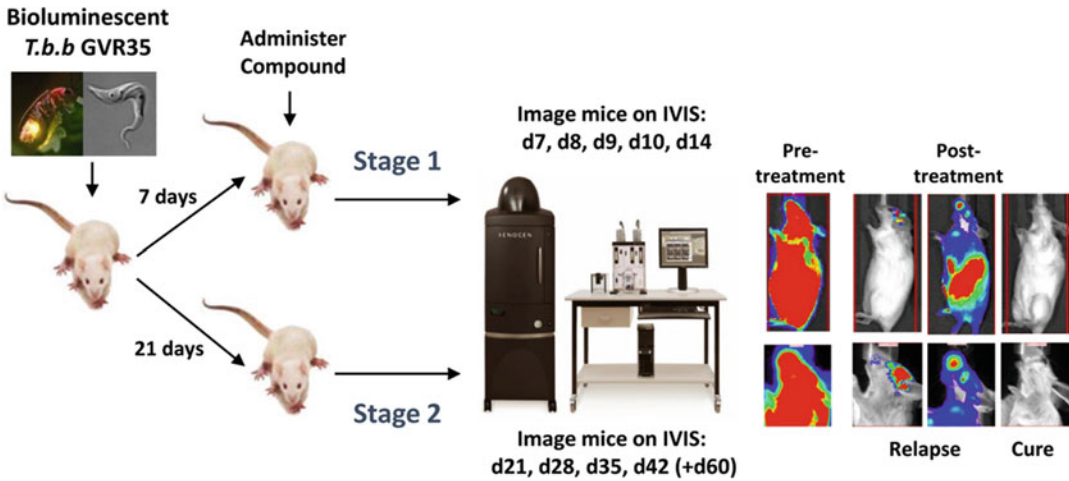
Fig. 1 Schematic of the *T. brucei brucei* GVR35-VSL2 imaging model. CD1 mice are infected with 3×10^4 bloodstream form *T. b. brucei* GVR35-VSL2 and treated with compounds from day 7 (d7) or day 21 (d21) postinfection to screen for activity during stage 1 or stage 2 trypanosomiasis, respectively. Whole body bioluminescence imaging is performed using an IVIS imaging system before treatment and daily (stage 1) or weekly (stage 2) after treatment to monitor parasite burden

2.1.2 Generation of Bioluminescent T. brucei *Stabilates*

1. Trypanosome dilution buffer (TDB): 20 mM Na_2HPO_4, 2 mM $NaH_2PO_4.2H_2O$, 80 mM NaCl, 5 mM KCl, 1 mM $MgSO_4.7H_2O$, 20 mM D-glucose, pH 7.4.

2. Adult, female CD1 (ICR) mice (~25 g body weight) maintained under specific pathogen free conditions (*see* **Note 3**).

3. 1 mL syringes and 25 G needles.

4. 0.9% NH_4Cl.

5. Blood lancets.

6. CBSS heparin solution: 25 mM HEPES, 120 mM NaCl, 5.4 mM $CaCl_2 \cdot H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 5.6 mM Na_2HPO_4, 11.1 mM D-glucose, pH 7.4, 10 units heparin/mL.

7. Light microscope and hemocytometer within the animal facility.

8. CO_2 euthanasia apparatus.

9. Fine bore polythene tubing (0.58 mm internal diameter, 0.96 mm external diameter).

10. Monoject aluminum hub blunt needles (0.6 mm $\times$ 25.4 mm).

11. Cryovial tubes.

12. Glycerol.

13. "Mr frosty" freezing container.

2.1.3 Testing of Bioluminescent T. brucei *Stabilates*	1. *Trypanosoma brucei brucei* GVR35-VSL2 [12] blood straws (*see* Subheading 3.1.2).

2. Trypanosome dilution buffer (TDB) (*see* Subheading 2.1.2).

3. Adult, female CD1 mice (~25 g body weight) maintained under specific pathogen free conditions (*see* **Note 3**).

4. 0.9% NH_4Cl.

5. Blood lancets.

6. 1 mL syringes and 25 G needles.

2.2 In Vivo Imaging Model

2.2.1 Infection of Donor and Experimental Mice

- Adult, female CD1 mice (~25 g body weight) maintained under specific pathogen free conditions (*see* **Note 3**).

- Blood straws of *T. b. brucei* strain GVR35-VSL2 (red-shifted) luciferase expressing cell line (*see* Subheading 3.1.2).

- Trypanosome dilution buffer (TDB) (*see* Subheading 2.1.2).

- CBSS heparin solution (*see* **item 7** in Subheading 2.1.1).

- 0.9% NH_4Cl.

- Blood lancets.

- 1 mL syringes and 25 G needles.

- Light microscope and hemocytometer in the animal facility.

- CO_2 euthanasia apparatus.

- Ear punch tool.

2.2.2 Bioluminescence Imaging

1. IVIS spectrum In Vivo Imaging System from PerkinElmer (*see* **Note 4**).

2. $MgCl_2$ and $CaCl_2$ free Dulbecco's PBS purchased from Gibco.

3. Beetle luciferin potassium salt (1 g) from Promega—Prepare 15 mg/mL working stock by resuspending 1 g beetle luciferin (D-luciferin) potassium salt in 66.66 mL of $MgCl_2$ and $CaCl_2$ free Dulbecco's PBS, freeze 1 mL aliquots at $-20\ ^\circ C$.

4. Isoflurane gaseous anesthesia equipment including gas vaporizer, scavenger, and oxygen generator (e.g., XGI-8 Gas Anesthesia System connected to an oxygen tank or generator).

5. Lacri-Lube ocular lubricant from Allergan pharmaceuticals.

6. PBS-G: $MgCl_2$ and $CaCl_2$ free Dulbecco's PBS with 10 mM glucose, pH 7.4.

2.2.3 Determine Blood Parasitemia

1. Blood lancets.

2. 0.9% NH_4Cl.

3. Light microscope and hemocytometer in the animal facility.

2.2.4 In Vivo Compound Treatments	1. Diminazene aceturate (control treatment).
	2. Distilled H_2O to solubilize diminazene aceturate.
	3. 0.22 μm syringe filters.
	4. 1 mL syringes.
	5. Mouse feeding tubes—plastic or metal.
	6. Lacri-Lube ocular lubricant from Allergan pharmaceuticals.
	7. PBS-G: $MgCl_2$ and $CaCl_2$ free Dulbecco's PBS with 10 mM glucose, pH 7.4.
	8. Test compounds and suitable vehicle.
	9. Weighing scales.

2.2.5 Blood Sampling for Pharmacokinetic Analysis	1. Blood lancets.
	2. Thermostatically controlled heated chamber.
	3. Mouse restraining apparatus.
	4. Heparinized tubes.
	5. Screwcap sample tubes.
	6. Antimicrobial solution (e.g., chlorhexidine).

2.2.6 End Point Imaging	1. In Vivo Imaging System (IVIS).
	2. Beetle luciferin (D-luciferin), 15 mg/mL working stock (*see* **item 3** in Subheading 2.2.2).
	3. PBS-G: $MgCl_2$ and $CaCl_2$ free Dulbecco's PBS with 10 mM glucose added, pH 7.4.
	4. Heparinized tubes.
	5. Dissection kit.
	6. Petri dishes.
	7. Liquid nitrogen and benchtop liquid nitrogen container.

3 Methods

3.1 Generation and Testing of Bioluminescent T. brucei *Stabilates*	The bioluminescence imaging model requires infection of donor mice from *T. brucei* blood stabilates. These blood stabilates can be generated from cultured parasites (Subheadings 3.1.1 and 3.1.2) or from F1 blood stabilate stocks that should be tested (Subheading 3.1.3) and then used for infection and generation of new blood stabilates (Subheading 3.1.2 from **step 5** onward) (*see* **Note 5**).

3.1.1 Culturing of T. b. brucei GVR35-VSL2 Bloodstream form Trypanosomes	1. Defrost a culture stabilate of *T. b. brucei* GVR35-VSL2 bloodstream form trypanosomes [12] and transfer the cell suspension to a 25 cm^3 vented flask containing 10 mL pre-warmed supplemented IMDM medium.

2. Leave to recover for around 72 h by culturing cells in vitro at 37 °C, 5% CO_2 in an upright flask.

3. Count cells and subpassage in supplemented IMDM to 5×10^4 cells/mL (*see* **Note 6**).

4. Following the first passage, count cells every 3–4 days and subpassage to ensure that the culture is maintained between 5×10^4 and 1×10^6 cells/mL (*see* **Note 7**).

5. Keep in vitro culture to a minimum to avoid effects on virulence.

3.1.2 Generation of Bioluminescent T. brucei *Blood Stabilates*

1. *For infection from cultured parasites*: Count cells of a log-stage *T. b. brucei* GVR35-VSL2 culture (between 5×10^5 and 1×10^6 cells/mL) to determine cell density.

2. Transfer 5×10^5 cells to a sterile tube, add 5 mL trypanosome dilution buffer (TDB) and centrifuge at $1500 \times g$ for 10 min at room temperature.

3. Carefully remove supernatant, repeat centrifugation as above in 5 mL fresh TDB.

4. Carefully remove supernatant, leaving ~500 μL in tube. Resuspend pellet in remaining TDB, count cells and adjust final concentration to 1.2×10^5 parasites/mL. Proceed to **step 6**.

5. *For infection from blood stabilates*: Defrost required amount of F2 blood straws for infection of two donor mice (determined in Subheading 3.1.3) into 500 μL TDB and mix well.

6. Inject two CD1 mice intraperitoneally with 250 μL (3×10^4 parasites) per mouse using a 1 mL syringe and 25 G needle (*see* **Note 8**).

7. Monitor parasitemia of infected mice daily as follows: Dilute 4 μL of blood (taken via a tail vein venipuncture using a lancet or needle, *see* **Note 9**) in 16 μL of 0.9% ammonium chloride to lyse the blood cells. Determine parasite density in blood by counting on a hemocytometer.

8. When parasitemia reaches the first peak (~1×10^7 parasites/mL) cull the mouse by exposure to CO_2 in a rising concentration, and remove all blood by cardiac puncture using a syringe pre-loaded with 200 μL of CBSS heparin buffer (this prevents coagulation of the blood) (*see* **Note 10**).

9. Add 71.4 μL of glycerol to every 500 μL of blood and mix with a vortex.

10. Using a 1 mL syringe and blunt needle inject blood/glycerol mix into fine bore polythene tubing and cut into ~2.5 cm sections, placing "blood straws" into cryovials.

11. Place cryovials in a "Mr Frosty" or cotton wool lined box and freeze at $-80\,^{\circ}$C overnight before transferring to liquid nitrogen for long-term storage (this is designated as the F1 storage stock).

3.1.3 Testing
of Bioluminescent T. brucei
Stabilates

1. Remove three frozen straws from liquid nitrogen and dilute in 500 μL TDB.

2. Count parasites with a hemocytometer to calculate the parasite number in one straw and the number of straws required to achieve a count of 1.2×10^5 parasites/mL.

3. Inject 250 μL (3×10^4 parasites) into a CD1 mouse by intraperitoneal injection.

4. Monitor parasitemia daily to calculate the length of time before the first parasitemia peak.

5. A second stock of blood straws (F2 working stocks) should be made from the F1 stock as in Subheading 3.1.2 (**steps 5–11**)—this F2 stock should also be tested as above and used for infections of donor mice for reproducible infections.

3.2   In Vivo Imaging Model

3.2.1 Infection of Donor and Experimental Mice

1. Select appropriate infection day of donor mice based on when mice are expected to develop the first peak of parasitemia (determined in Subheading 3.1.3) (*see* **Note 11**).

2. Defrost required amount of F2 blood straws for infection of two donor mice (determined in Subheading 3.1.3) into 500 μL TDB and mix well.

3. Inject each mouse intraperitoneally with 250 μL of TDB/blood straw mixture.

4. Monitor parasitemia daily as described in Subheading 3.1.2 (**step 7**) until the first peak of parasitemia is achieved (5×10^6–1.0×10^7 parasites/mL).

5. Harvest blood by cardiac puncture under terminal anesthesia using a syringe prefilled with 200 μL of CBSS heparin to prevent coagulation of the blood.

6. Determine the blood parasitemia and dilute blood with CBSS heparin to achieve a concentration of 1.2×10^5 parasites/mL before injecting each experimental mouse with 250 μL (3×10^4 parasites/mouse).

7. Ear mark mice for identification and weigh all mice.

8. Assign mice to treatment groups (*see* **Note 12**).

3.2.2 Bioluminescence Imaging (See **Note 4***)*

1. At day 7 (stage 1 model) or day 21 (stage 2 model) postinfection (*see* **Note 13**) image mice before treatment using the IVIS spectrum as described below. Posttreatment imaging should be done daily or weekly after start of treatment (*see* **Note 14**). Determine blood parasitemia (*see* Subheading 3.2.3) on the same day as imaging.
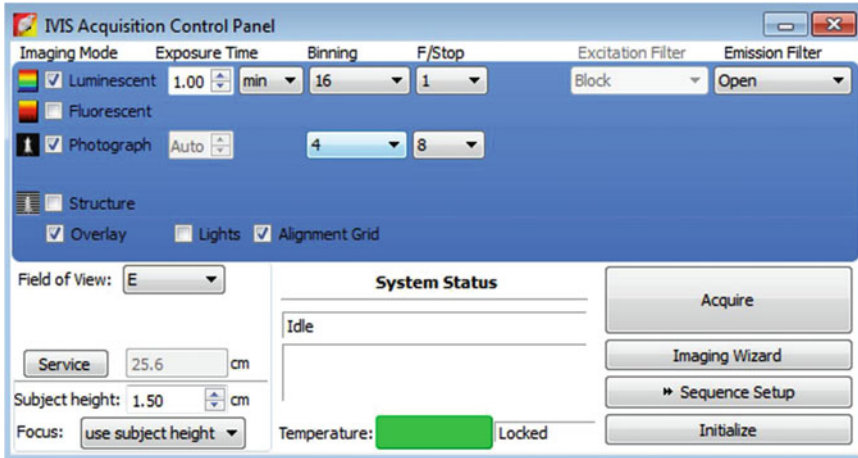
Fig. 2 IVIS Acquisition Control panel in the Living Image Software. An example of the image settings for acquisition of bioluminescence is shown. In this case, exposure time of 1 min, binning of 16, F/stop of 1 and field of view E is selected for imaging of the whole body after compound treatment (*see* Table 1)

2. Defrost the appropriate number of beetle luciferin (15 mg/mL working stock) vials (10 μL/g of mouse is required).

3. Start the Living Image software. Enter initials or select from the list when prompted to do so by the software.

4. Create an appropriate folder on the computer hard drive to save the data generated.

5. Once the program opens initialize the system using the "Initialize" tab in the bottom right corner of the control panel (Fig. 2).

6. Once the system is initialized the "System Status" in the control panel will show "Idle" and the "Temperature" box will be green (Fig. 2, bottom middle).

7. Place a black XFM-1 low fluorescence mat inside the imaging chamber with the matt side facing upward (the black mat is required for bioluminescence and fluorescence imaging but may have been removed by others when doing trans-illumination imaging).

8. Start the anesthesia/isoflurane system—this should be connected to the IVIS and to an induction chamber. Make sure that the system receives O_2 (for example from an open O_2 tank or oxygen generator) and that the isoflurane level is full. If not then these will need to be filled/replaced before proceeding.

9. Turn on the O_2 supply and the waste gas scavenger.

10. Transfer 3–5 mice to the induction chamber and close it tightly (*see* **Note 15**).

11. Initiate anesthesia using 1.5 L per minute O_2 with 4% isoflurane. Ensure that the flow of isoflurane is toward the induction chamber.

12. Inject 150 mg/kg beetle luciferin subcutaneously (using 10 μL of the 15 mg/mL stock per gram of mouse) and record the time of luciferin injection (*see* **Notes 16** and **17**).

13. Mice are usually imaged from 10 to 12 min after luciferin injection (*see* **Note 18**)—while waiting for this time to pass choose the appropriate predetermined settings on the Living Image software control panel (Fig. 2, top and left panels). For imaging in this model and using subcutaneous injection of luciferin we use the settings shown in Table 1. The time of image acquisition and sequence of images are shown below:

    Time of image acquisition: start 12 min after luciferin injection (the bioluminescent signal plateau is from 15 to 20 min).

    Sequence of imaging: (1) one ventral whole body image, (2) one side whole body image (with spleen facing upward), mice are then imaged individually for (3) one dorsal head image, (4) one side head image (*see* **Note 19**).

    Ensure that the "Overlay" and "Alignment Grid" boxes (middle of control panel) are ticked.

14. Using a cotton swab, gently apply Lacri-lube ocular lubricant directly to the eyes of each mouse to prevent eyes from drying out.

15. Transfer mice to the IVIS imaging chamber and place each mouse with its nose in a nose cone; ensure that unused nose pieces are closed with black plugs (*see* **Note 21**).

Table 1
Image acquisition settings used for the *T. b. brucei* GVR35-VSL2 imaging model

Field of view (FOV)	Imaging mode	Exposure time	Binning	f/stop	Excitation
Untreated mice/whole body					
E	Luminescence	1 s	Small (4)	1	Block
Untreated mice/head					
A	Luminescence	1 s	Small (4)	1	Block
Treated mice/whole body (*see* **Note 20**)					
E	Luminescence	1 min	Large (16)	1	Block
Treated mice/head					
A	Luminescence	1 min	Large (16)	1	Block

16. Place light absorbing dividers between mice to avoid signal contamination (*see* **Note 22**).

17. Reduce isoflurane to 1.5% and direct the flow of anesthetic to the IVIS system (using the ON/OFF switch or turning the knob toward the IVIS system—this mechanism depends on the system being used).

18. Acquire images by clicking "Acquire" on bottom left side of the control panel.

19. Upon acquisition of the first image after initialization the software will offer the option of auto-saving and allow selection of the location for the saved files. Select auto-save.

20. Various input boxes are available to record data, such as time point, mouse strain and general comments. Useful information to record are the treatment group, mouse numbers, time-point, type of image (e.g., ventral body or dorsal head), and the luciferin injection time (this allows easier selection later of images that were acquired at exactly the same time after luciferin injection).

3.2.3   Determination of Blood Parasitemia

1. Dilute 4 μL of blood (taken via a tail vein venipuncture using a lancet or needle) in 16 μL of 0.9% ammonium chloride to lyse the blood cells. Determine parasite density in blood by counting on a hemocytometer.

3.2.4   In Vivo Compound Treatments

1. The optimal vehicle and dosing route for drug treatment should be determined in advance (*see* **Note 23**). Diminazene aceturate is administered intraperitoneally in water as a single dose at 40 mg/kg in distilled water.

2. Prepare the vehicle and compounds one day before treatment (if compound stability allows for this). Diminazene aceturate should be prepared fresh on the day of treatment and filtered using a 0.22 μm syringe filter.

3. Store treatment compounds as aliquots at 4 °C during treatment regime. Bring to room temperature and mix well before each treatment.

4. Treatment starts at day 7 (stage 1 model) or day 21 (stage 2 model) after infection (*see* **Note 13**); mice should be weighed and dosed according to weight (*see* **Note 24**).

5. If required take blood samples for pharmacokinetic analysis as described in Subheading 3.2.5.

6. Weigh mice and monitor daily for adverse effects of infection or treatment.

3.2.5 Blood Sampling for Pharmacokinetic Analysis

1. Blood sampling (site, volume and frequency) should be done in accordance with animal welfare legislation and as approved by the relevant Ethics Committee. If needed this may require additional animals or alternating bleeding of mice to minimize stress and stay within ethical guidelines.

2. Prepare labeled collection tubes in advance.

3. Heat mice in a thermostatically controlled warm air box to dilate blood vessels (in this case, the tail vein).

4. Disinfect the blood sampling site by washing with an antimicrobial solution (e.g., 2% water-based chlorhexidine).

5. Restrain the mouse and puncture the skin and underlying blood vessel using a needle or lancet.

6. Withdraw the appropriate amount of blood into a heparinized collection tube.

7. Stop blood flow by applying finger pressure on the site for ~30 s and return the animal to its cage.

8. Mix the blood in the collection tube, transfer the appropriate volume(s) to fresh nonheparinized tubes and freeze at $-20\,^{\circ}C$ for pharmacokinetic analysis.

3.2.6 End Point Imaging

1. For the stage 1 model the end point may range between day 14 and 21. For the stage 2 model the untreated controls should be culled at day 28–30 postinfection (*see* **Note 25**), while diminazene aceturate controls and treatment group mice will be culled upon relapse of parasitemia in blood. If no relapse occurs in the treated group, mice may be monitored for longer periods until a defined end point (e.g., day 100 or day 180).

2. At the chosen end point image mice as described in Subheading 3.2.2.

3. Cull mice by cervical dislocation or exposure to a rising concentration of CO_2 (the latter is advised if perfusion of mice is required).

4. Optional: Perfuse mice with PBS-G to remove all blood from the brain for quantification of the brain-resident parasites.

5. Remove the brain and place into a petri dish; add 100 μL of beetle luciferin (15 mg/mL stock) onto the brain surface and image ex vivo, using 1-min exposure time, large binning and f stop 1. The signal peaks ~8 min after luciferin application; however, images are taken continuously up to 10 min after application.

6. Optional: Rinse brains with PBS, snap-freeze in liquid nitrogen and store at $-80\,^{\circ}C$ for qPCR analysis [13, 16].

3.3 Image Analysis	1. Analyze images using Living Image software from PerkinElmer as described in the software manual.

4   Notes

1. All animal procedures described in this chapter are in accordance with the guidelines of the UK Home Office under authorized Home Office licenses and approved by the relevant Animal Welfare and Ethics Committees. Its implementation in other laboratories will require approval from relevant government and institutional committees before commencing work.

2. *T.b.b.* GVR35-VSL2 lines can be obtained from the corresponding author as culture stabilates or as blood stabilates for infection of donor mice.

3. Outbred CD1 (ICR) mice tolerate infection with *T. brucei* GVR35 well. Pilot studies with inbred BALB/c mice indicated that this strain is more susceptible to secondary infections when infected with *T. brucei*.

4. The GVR35 imaging model described here makes use of bioluminescence imaging (Subheading 3.2.2) with the IVIS spectrum imaging system (PerkinElmer) and Living Image analysis software (Subheading 3.3); the model can also be adapted for use with other in vivo small animal imaging systems and analysis software.

5. In vitro culturing will affect the virulence of *T. brucei* GVR35; use of F1 blood stabilates for the generation of new blood stabilate working stocks is recommended.

6. Cells may grow very slowly after the first passage. Some cells will die while others continue to divide resulting in no net increase in cells in the first couple of days. The culture will recover and grow normally (~three fold overnight) between 1 and 2 weeks after thawing of the stabilate. Cells will start growing faster (growth of fivefold overnight) over time but may also become more virulent so this should be avoided to ensure reproducibility of the model.

7. The cells will start dying from $1.5–2 \times 10^6$ cells/mL so are best kept at a density below 1×10^6 cells/mL.

8. Mice will develop parasitemia at different times and parasites harvested from blood may vary in their infectivity. It is therefore advised to infect two mice for harvesting of parasites and to make two sets of stabilates for later selection of the set which results in the most reliable infections (Subheadings 3.1.2 and 3.1.3).

9. Single-use needles and lancets should be used to avoid contamination and additional injury to mice.

10. It is important to harvest parasites from mice while parasitemia is increasing. Once parasitemia has reached its peak and decreases again the parasites will be less infective. Harvest parasites when parasitemia is in the range of 5×10^6–1.5×10^7 parasites/mL of blood.

11. Parasites will be harvested from donor mice during their first peak of parasitemia and then used to infect experimental mice (designated as day 0). Imaging and treatment of mice is usually done from day 7 (stage 1 model) or day 21 (stage 2 model) after infection and should ideally fall on a convenient day that will also allow for preparation of compounds one day in advance. For example, our blood straws produce an infection in donor mice with the first peak of parasitemia developing 4–5 days postinfection. By infecting donor mice on a Thursday blood and parasites can usually be harvested on the following Tuesday for infection of experimental mice. This allows for preparation of compounds on Monday and imaging as well as start of compound treatment on Tuesday.

12. We routinely set mice up in groups of six per treatment with three diminazene aceturate-treated controls and three untreated controls. These animal numbers have been determined using a power analysis with variables estimated from previous experiments. The variables and calculated group sizes may change depending on the mice used, the bioluminescence of the parasites and sensitivity of the imaging systems. Pilot studies should be performed when setting up a new model to estimate the variables for power analysis.

13. The GVR35 mouse model mimics stage 1 disease at day 7 postinfection. By day 21 the parasites become established in the CNS and infections can only be cured by compounds active in the brain [16, 17]. For screening in stage 2, mice should always be treated after 21 days of infection. Treatment can also be delayed by 1 or 2 days if required for logistical reasons (e.g., day 8 or 9 for stage 1 and day 22 or 23 for stage 2) with subsequent delay in the posttreatment imaging time points.

14. The imaging schedule should be determined in advance and will depend on what is feasible and what analysis is to be done. To analyze in vivo kill kinetics daily imaging after treatment may be needed. For stage 1, imaging usually starts on day 7 and can be done daily afterward up to day 14 (e.g., day 7, 8, 9, 10, 14). For stage 2, imaging usually starts at day 21 and is done weekly, or later (usually after day 63), 2-weekly intervals following start of treatment (e.g., day 21, 28, 35, 42, 49, etc.).

15. The number of animals to be imaged will depend on the IVIS imaging system being used. We prefer to image three mice at a time to allow space between mice.

16. It is preferable to inject luciferin subcutaneously or intraperitoneally. While subcutaneous injection results in a slightly lower signal compared with intraperitoneal injection it has a longer and flatter signal kinetics making it more suitable for taking multiple images of the whole body or head. Furthermore, there is a higher risk of failure for intraperitoneal injection (where injection is placed into a site other than the peritoneal cavity, such as the lumen of the stomach or bowel, or injection is subcutaneous, retroperitoneal or intravascular), particularly in trypanosome-infected mice where spleens are enlarged. A failed injection may reduce the luciferin concentration in tissues resulting in lower bioluminescence or result in death.

17. Luciferin can be injected once the animal is anesthetized; however, this will alter the kinetics of the signal compared with an animal that is awake. This should be taken into account when establishing acquisition timings and setting.

18. Imaging acquisition settings and the timing of imaging after luciferin injection need to be determined in advance. This will depend on the site of luciferin injection, strength of the signal, and location of the signal. To establish the imaging start time calculate a kinetic curve during a pilot study—this is done by taking multiple images over time and determining when the signal peaks and falls.

19. The mouse to be imaged is moved to the middle nose cone while the other two remain on outer nose cones. For head images the "Field of view (FOV)" on the IVIS acquisition panel should be set on A—this increases resolution by moving the stage upward but will only allow imaging of the middle mouse (e.g., for the IVIS spectrum FOV A is 3.9×3.9 cm). Once head images of the middle mouse are completed it is transferred to a holding box and another mouse is moved to the middle for imaging of head (any open nose cones should be blocked with black plugs as mice are transferred from the imaging chamber).

20. Mice may have low bioluminescence signal after treatment with a trypanocidal compound; to detect these low signals increase the exposure time and the binning. In untreated mice, or mice with relapsed parasitemia, signal may be saturated at high exposure and binning; in these instances the exposure time and binning should be decreased or the F/stop increased.

21. Transparent nose cones ensure gas delivery to individual mice from the anesthesia manifold. Black rubber plugs seal unused ports of the anesthesia manifold. Nose cones and plugs should be provided with the IVIS gas anesthesia system.

22. XMD-2 manifold dividers are available in different heights and can be purchased from PerkinElmer or may have been provided with the IVIS system. We cut dividers from black Lexan plastic sheets. In cases where high signal from a mouse still spills over to other mice with low signal it may be necessary to re-image mice separately (e.g., this may occur when parasites have recrudesced in one mouse while others remain clear of parasite signal).

23. The vehicle and dosing regimen for compounds should be determined in advance and tested for toxicity in uninfected mice before used for treatment in the *T. b. brucei* infection model.

24. Compound administration should be done in volumes as specified in the appropriate animal project license or as recommended by animal research advisory boards. For example for oral dosing it is considered good practice to administer compounds in 10 mL vehicle/kg of mouse [18].

25. CD1 mice infected with *T. b. brucei* GVR35 may develop symptoms related to infection of the CNS such as hind-leg paralysis and paresis. This usually occurs from day 28–35 post-infection. To avoid these adverse effects from developing untreated mice should be culled at day 28–30 postinfection (e.g., after the imaging session, 7 days after start of treatment) or when symptoms become visible.

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