TITLE: Bioluminescence imaging to detect late stage infection of African trypanosomiasis

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KEYWORDS:
Bioluminescence; firefly luciferase; CNS; optical imaging; trypanosomes; blood-brain barrier; mouse model; preclinical research.

SHORT ABSTRACT:
This manuscript describes the use of a bioluminescent strain of African trypanosomes to enable the tracking of late stage infection and demonstrates how *in vivo* live imaging can be used to visualize infections within the central nervous system in real-time.

LONG ABSTRACT:
Human African trypanosomiasis (HAT) is a multi-stage disease that manifests in two stages; an early blood stage and a late stage when the parasite invades the central nervous system (CNS). *In vivo* study of the late stage has been limited as traditional methodologies require the removal of the brain to determine the presence of the parasites.

Bioluminescence imaging is a non-invasive, highly sensitive form of optical imaging that enables the visualization of a luciferase-transfected pathogen in real-time. By using a transfected trypanosome strain that has the ability to produce late stage disease in mice we are able to study the kinetics of a CNS infection in a single animal throughout the course of infection, as well as observe the movement and dissemination of a systemic infection.

Here we describe a robust protocol to study CNS infections using a bioluminescence model of African trypanosomiasis, providing real time non-invasive observations which can be further analyzed with optional downstream approaches.

INTRODUCTION:
Human African trypanosomiasis (HAT), or sleeping sickness, is caused by the vector-borne protozoan parasites of the *Trypanosoma brucei* spp[1]. Estimated numbers of current cases is fewer than 7 thousand every year with almost 70 million people exposed to the risk of the

parasite infection within the African continent. The disease, which is most often lethal if left untreated, comprises an early hemolymphatic stage where parasites are present in the blood, progressing to the late stage when parasites invade the central nervous system (CNS) and are no longer susceptible to treatment by early stage trypanosomal drugs[2]. The current drug therapies for late-stage HAT have both complex, prolonged, treatment regimens and severe adverse effects as well as reported resistance, therefore research into new drug therapies is imperative[3,4].

The study of late-stage human African trypanosomiasis (HAT) within traditional mouse models is lengthy and complex, with the removal of brain tissue being required to monitor parasitic burden[5]. The animal infective strain *T. b. brucei* is used as the study model of trypanosomiasis with the late stage appearing 21 days post infection (dpi). To monitor the wild type nonbioluminescent parasite infection in the mouse model, peripheral blood films or quantitative PCR are the only methods to determine parasite burden. For parasite burden in the brain, the mouse needs to be culled, brain excised and qPCR carried out on tissues, making it impossible to track parasites through multiple time points in the late stage infection. This results in the inability to follow real-time infections within the central nervous system (CNS).

In vivo bioluminescence imaging (BLI) can provide highly sensitive, non-invasive detection of parasite dissemination and disease progression in a mouse model that can be followed in a single animal for the entirety of the experiment[6]. BLI is based on the emission of light in the visible spectrum produced by a luciferase-catalyzed reaction. The emitted photons are then detected by a charge coupled device (CCD) camera[7]. For this purpose, the pathogen is genetically modified to express a luciferase protein and the substrate, luciferin, is introduced at time points of interest by injection. The main advantage of this method is the ability to carry out longitudinal studies, in which the same animal can be imaged several times with minimal adverse effects. The acquired bioluminescence signal can be quantified, thus indicating the pathogen burden.

The optimization and validation of a red-shifted bioluminescent *T. b. brucei* has enabled the investigation of the late stage infection through non-invasive procedures, detecting parasites earlier than blood film microscopy and greatly reducing the time, cost and numbers of animals needed to study CNS infection and drug screening in late-stage trypanosomiasis[8,9]. In this protocol we demonstrate infection of mice with bioluminescent trypanosomes and how to then visualize the parasites *in vivo* for quantification of disease progression and CNS penetration.

PROTOCOL:

Ethics

All work was carried out under the approval of the UK Home Office Animals (Scientific Procedures) Act 1986 and the London School of Hygiene & Tropical Medicine Animal Welfare and Ethics Review Board. ARRIVE guideline are followed in this report.

1. *In vivo* passage of bioluminescent *Trypanosoma brucei brucei*

1.1) Remove a cryopreserved stock (termed stabilate) of *T. b. brucei* strain GVR35-VSL-2 (available from Professor John Kelly at the London School of Hygiene & Tropical Medicine through MTA)[9] from liquid nitrogen and allow to equilibrate to room temperature. Dilute stabilate to 2 x 10^4 trypanosomes/ml in sterile phosphate buffered glucose saline (PBS-G) pH 8.0 (1 litre PBS + 15 g glucose)[10].

1.2) Inject a 20-25 g female CD1 mouse with 0.2 ml diluted trypanosomes via the intraperitoneal route (i.p.) with a 25G needle. This is the "donor mouse". Place the infected mouse into a Specific Pathogen Free (SPF)-cage and feed *ad libitum*.

Note: CD1 mice are an outbred strain and *T. b. brucei* GVR35 infections are well-characterized in this strain. However, this protocol is also applicable to inbred or genetically-modified strains, but it is important to note that fur color may impact on the sensitivity of imaging[6,9].

1.3) Monitor peripheral parasitemia by blood sampling. Place the mouse into a plastic restrainer and take 5 µl of blood from the tail by venepuncture with 21G needle or venesection and mix 1/10 with 0.85% sterile ammonium chloride to lyse red blood cells and allow easier counting.

1.4) Count the diluted blood in a Neubauer hemocytometer.

1.4.1) Load the diluted blood into both chambers. Count the entire central grid of one chamber to give *n* trypanosomes. Concentration of trypanosomes in blood = n x 10^4 trypanosomes/ml, adjust for any dilution factor and calculate the average of the two chambers.

Note: After approximately 5-7 days post-infection, peripheral parasitemia should rise sufficiently to carry out an infection at a concentration of 2 x 10^4 trypanosomes/ml.

1.5) When trypanosome levels are high enough for the number of mice required, move on to step 1.6. A parasitemia level of 6 x 10^4 trypanosomes/ml in one donor mouse is sufficient for infection of 10 mice.

Note: For statistically significant group sizes, n = 6 for each treatment group of mice are needed[8]. For other models of infection, power calculations would need to be applied to determine appropriate group sizes.

1.6) Place mice into a heat box (at 28-30 °C for approximately 5-10 minutes) to allow the tail veins to dilate. To induce terminal anaesthesia, administer 20 mg/kg pentobarbital i.v with a 25G needle. Confirm mouse is anesthetized by gently pressing the footpads to ensure there is no withdrawal reflex.

1.7) Heparinize a 21G needle by aliquoting 5 ml heparin at 5000 USP units/ml into a Bijou tube and sucking twice in and out of the needle attached to a 1 ml syringe.

1.8) Take the heparinized 21G needle and 1 ml syringe, and perform cardiac puncture on the anesthetized passage mouse by inserting the needle centrally underneath the rib cage (a bead of blood will pool in the base of the needle) and gently pull back on the plunger so as not to collapse the chamber of the heart. Collect a minimum of 0.7 ml of blood.

1.9) Dilute infected blood to produce an inoculum of 2×10^4 trypanosomes/ml in PBS-G pH 8.0 as step 1.1 above.

Note: At this point, the remaining blood can be cryopreserved to produce stabilate by mixing with 8% glycerol, 20% heat-inactivated foetal calf serum (hi-FCS) and 72% infected blood. Slowly freeze at -80°C using a slow-freeze freezing container to achieve a cooling rate of -1°C/minute, before transferring to liquid nitrogen.

2. Infection of experimental mice

2.1) Place CD1 female mice (approx. 21-25 g) into a heating box to gently warm to dilate veins. Place the warmed mice into a plastic restraint and inject 0.2 ml diluted inoculum intravenously with a 25G needle into the tail vein, equivalent to 4000 trypanosomes per mouse. Place pressure at the injection site afterwards to stem the bleeding.

Note: Intraperitoneal infection is a valid route of infection and has been used in previous studies[10], but we have found this less reproducible than the intravenous route.

2.2) Randomize infected mice and cage in groups of 3 in SPF-vented cages. As a control, inject CD1 female mice with wild type non-bioluminescent parasite, *T. b. brucei* GVR35 (*n* = 3).

2.3) Monitor the infection through blood film microscopy

2.3.1) Spot 5 µl blood from venepuncture or venesection onto a glass slide and perform a blood smear (using a second glass slide gently push the drop of blood across the slide to produce a thin film). Allow slides to air dry.

2.3.2) Fix the blood smear with 100% methanol and stain with Giemsa for 10 minutes before rinsing with dH_2O and allow to air dry. Under x100 objective count the number of trypanosomes in 10 fields of view (f.o.v.).

Note: Blood filming is carried out alongside whole animal noninvasive imaging as early as one day post-infection. When processing a number of animals, this method of trypanosome quantitation is preferred over hemocytometer counting as the samples can be stored at room temperature and counted later.

3. Bioluminescence imaging to track infection

Note: To monitor the infection, whole animal non-invasive imaging can be used.

3.1) Prepare a stock solution of D-luciferin, the firefly luciferase substrate, in Dulbecco's Phosphate Buffered Saline (DPBS) at 30 mg/ml. Filter sterilize and freeze in aliquots at -20°C.

Note: D-Luciferin is light sensitive, therefore protect it from light by wrapping aliquots in foil. Do not repeatedly freeze-thaw luciferin as it can affect activity[11].

3.2) Warm an aliquot of D-luciferin to room temperature. Remove each mouse from the cage (including control mice infected with nonbioluminescent wild type parasite strain) and inject 150 mg/kg of the warmed D-luciferin (diluted in DPBS) intraperitoneally with a 25G needle. Place the mice into an isofluorane chamber for approximately 5 minutes to induce anesthesia with 2% isofluorane/O_2 mix at 2.5 L/min.

Note: Mice become immobile when they are under anesthesia. For the imager used here, 3 mice can be processed at a time. If mice are anesthetized for longer than 30 minutes, ophthalmic artificial tear ointment can be applied to prevent the eyes of the mice from drying out.

3.3) For the imager described here, open the software and initialize the machine using the software as per manufacturer's instructions. Acquire images once camera and heated plate have reached temperature.

Note: This instrument is not usually switched off, enabling the machine to perform background calibrations overnight. In the event that it needs to restart, it must run a calibration check first.

3.4) Place the anesthetized mice into the imager (see 3.5). Use large black separators between the mice to minimize bleed through of any bright bioluminescent signal. Image mice using a set of exposure times; 1, 3, 10, 30, 60 and 180 seconds, with medium binning, 1 f/stop and an open filter, and field of view E (12.5 x 12.5 cm).

Note: The control mice infected with the wild type nonbioluminescent GVR35 parasites provide a background bioluminescence value. From a luciferin kinetics experiment (Figure 6) with this model, we have found that bioluminescence signal peaks at 10 minutes post administration and imaging takes approximately 5 minutes for 3 mice. Take this timescale into consideration when anesthetizing and imaging the mice.

3.5) To ensure thorough imaging of the mice, rotate to obtain a ventral, dorsal and lateral view. Maintain consistency in the order of orientation for imaging between sequential animals.

3.6) After imaging, allow mice to recover from anesthesia under observation before returning to SPF-cage.

3.7) Continue imaging mice every 7 days until day 21 post-infection. There should be a steady increase in bioluminescence signal over the entire animal.

3.8) At D21, image and blood film the mice as described above, and dose mice with 40 mg/kg diminazene aceturate intraperitoneally. This drug will clear the peripheral parasitemia but will not cross the blood-brain barrier, and therefore enables better visualization of the CNS infection.

Note: Diminazene aceturate is a well-established drug used to treat early-stage animal trypanosomiasis.

3.9) Continue imaging and monitoring at D28 and D35.

4. Confirming CNS infection

4.1) At D35, image mice as detailed above (steps 3.1-3.4).

4.2) After imaging, allow mice to recover from anesthesia and exsanguinate mice as detailed in step 1.6-1.8, collecting blood for further analysis.

4.3) Following cardiac puncture, perfuse mice with 20 ml PBS (optional: add 3 mg/ml of luciferin to perfusion solution) via the heart to clear blood from the organs and to reintroduce luciferin label that might have cleared from the brain region over the time interval from step 4.1.

4.4) Gently remove the brain, by using curved scissors, cutting from the base around the circumference of the skull, and lifting off the top part of the skull, to allow the brain to be gently lifted out with curved forceps.

4.5) Place the excised brain onto a section of black plastic and pipette 50 µl of 15 mg/ml luciferin over the organ prior to imaging, to ensure adequate luciferin has been added to the excised brain. Image using the settings as above. This will demonstrate localization of the infection to the brain hemispheres.

Note: Downstream quantification of parasitemia on the excised brain can be carried out by qPCR as previously described[8]. Other alternative downstream applications might include histology or immunohistochemistry to identify parasites in the brain tissue following fixation.

5. Quantitation of bioluminescence imaging

Note: Bioluminescence can be quantified using the region of interest (ROI) with the imaging software and corrected for background bioluminescence.

5.1) Using the ROI tool, create a rectangular ROI for whole animal quantitation and position it over the mouse image to cover tip of the nose to base of the tail of the mouse. Use the same size box for every animal and every time point. When looking at the ROI for the brain, use a circular ROI in the same way, positioning it over the mouse head region in the image.

5.2) If desired, adjust images to appear on the same spectrum for a visual representation of infection.

Note: The software used here generates a color map for each image automatically adjusted for minimum and maximum photon counts. To enable visual comparison between a series of images, the color scale must be set to the same values for all images.

5.2.1) Using the image with the highest bioluminescence and the 'Image adjust' tab on the tool palette, select a logarithmic scale and an appropriate color scale minimum and maximum (to be determined empirically). Apply this scale to all images throughout the experiment.

REPRESENTATIVE RESULTS:

This protocol demonstrates how to follow disease progression following infection of mice with *T. b. brucei*, a model for human African trypanosomiasis. **Figure 1** shows the timeline of the experimental protocol, demonstrating the timetable for treatment and imaging steps. **Figure 2** demonstrates a typical field of view in a fixed Giemsa-stained blood smear used to quantitate peripheral parasitemia, with trypanosomes and red blood cells present. Development of the infection in animals can be followed by measuring the bioluminescence as in **Figure 3**, which shows the infected animals over the course of an experiment with the same animals imaged at each time point. Through the course of infection an increase in bioluminescence can be observed in all the animals over the first 21 days as the signal becomes more disseminated and intense, with high regions of BLI (represented as red from the heat map scale) in the spleen area. At D21 mice are treated with diminazene aceturate as detailed in Figure 1, post-imaging, and a drop in bioluminescence can be observed at D28 as the peripheral infection is cleared, with low levels of signal being present in the head region of the mice. At D35 the signal still remains in the head region and has become more intense indicating a possible brain infection. The brain is excised to confirm signal is present within the brain tissue and can then be quantitated by qPCR.

Traditionally, blood smears are used to quantitate infection progression and treatment effects. However, these only measure peripheral parasitemia, and cannot be used to evaluate parasite levels in the brain. **Figure 4** combines quantitation of both the peripheral parasitemia and bioluminescence to demonstrate disease kinetics. At D7 a high BLI signal is present of 10^8 photons/second, but the parasitemia is very low within the blood films. Both the parasitemia and signal then increase and at D21 the parasitemia drops but bioluminescence remains constant which is in part due to the trypanosomes moving into the lymphatic system and tissues and also due their host-evasion method of switching their surface glycoproteins (VSG). After D21 the parasitemia becomes undetectable in the blood films due to the treatment with diminazene aceturate but, despite the initial dip in bioluminescence, the signal is still above background and continues to D35, a measure of the bioluminescence in the head region seen in Figure 3. This demonstrates the greater sensitivity of the bioluminescence approach over traditional blood film counting.

To verify that the bioluminescence in the head region is truly localizing to brain tissue, at the end of the experiment the brains are excised and bioluminescence measured *ex vivo* (**Figure 5**). A difference in infection burden between brains is often seen and signal does not appear

to localize to specific anatomical regions as distribution of intense BLI signal can vary between infected animals. For example, strong BLI signal is present in the olfactory bulb in mouse 2 and 3 and in the cerebellum in mouse 3, while the brain from mouse 1 shows general distribution of BLI signal all over the brain. As the brains can be imaged immediately after culling the mice, further analysis can be carried out on the brain tissue as the tissue is not damaged in the *ex vivo* imaging process.

Figure Legends:

Figure 1: Timeline of experiment including imaging, sampling and treatment. Due to the non-invasive nature of bioluminescence imaging, monitoring of infection can occur more often than detailed in the diagram. The sampling and imaging every 7 days enable the identification of the progressing infection. The treatment at D21 with diminazene aceturate removes peripheral parasitemia to allow better visualization of the head and brain infection. Diminazene aceturate is an early stage drug and is not able to pass the blood-brain barrier in quantities adequate to clear the late stage infection.

Figure 2: Giemsa stained trypanosomes. On a fixed blood film, Giemsa stained trypanosomes (purple with pink nuclei, closed arrow) are easily detectable against the red blood cells (stained blue, open arrow) and can be quickly counted to obtain the number of trypanosomes/ 10 fields of view under a 100x objective. The scale bar denotes 10 µm.

Figure 3: Bioluminescence imaging of infected mice. Three representative animals of a group of 6 mice infected with VSL-2 (M = mouse) and a wild type infected control (infected with the wild type nonbioluminescent parasite line). Luciferin was also administered to the control mouse prior to imaging, which provides the background measurement. A heat map scale is shown with red indicating high levels of bioluminescence, equating to high numbers of parasites, and blue indicating low levels of bioluminescence (D = day). The control mouse has no bioluminescence as expected with no luciferase-expressing parasites. Image from Burrell-Saward *et al*[8] by permission of Oxford University Press.

Figure 4: Comparison of bioluminescence and blood film parasitemia. Total bioluminescence of each whole animal was quantitated as described in section 5 and peripheral parasitemia quantitated by counting parasites in blood smears. Error bars denote standard deviation from the mean. Image from Burrell-Saward *et al*[8] by permission of Oxford University Press.

Figure 5: *Ex vivo* imaging of excised brain. The perfused brains of the mice imaged in Figure 3 were removed and imaged as described in 4.5. Bioluminescence is measured on a heat map scale as for Figure 3. The control brain from a mouse infected with wild type nonbioluminescent parasites was also treated with luciferin and no bioluminescence can be observed. The scale bar denotes 1 cm. This figure has been modified from Burrell-Saward *et al*[8] by permission of Oxford University Press.

Figure 6: Kinetics of luciferin bioluminescence in VSL-2-infected mice. Three VSL-2-infected CD1 mice were injected with 150 mg/kg i.p of luciferin and placed immediately within the imager. The mice were imaged over 60 minutes and their bioluminescence was calculated to

determine the optimum imaging window after luciferin administration.

DISCUSSION:

The development of a bioluminescent *T. b. brucei* GVR35 strain allows the visualization of a trypanosome infection from the early to the late stage. Previous infection models were unable to detect the late stage, when parasites are in the brain, in real time from blood film microscopy, and required the culling and removal of brains from the infected mice to determine parasite burden[12]. The bioluminescence reduces inter-mouse variability as a single mouse can be tracked throughout the entirety of the infection and a quick qualitative assessment of infection can be observed at any stage. The highly sensitive method of bioluminescence imaging enables low-level infection to be identified earlier than blood-film microscopy, with as few as 100 trypanosomes detectable through imaging compared to the 5×10^3 trypanosomes/ml detectable through microscopy[9]. In addition, following the same animals throughout the experiment eliminates the need for separate animal groups for each time point, greatly reducing animal usage.

The imaging protocol described here can be adapted to suit the scientific need as the non-invasive nature means that additional and more frequent imaging can be carried out (with appropriate animal ethics approval), but for the assessment of brain infection D21 is the earliest time point. At this point, early stage drugs are no longer effective at clearing the late stage parasites. It is also imperative that the mice are imaged under the same conditions at each time point. For example, the same exposure times and same view (ventral or dorsal) should be used to ensure accurate measurement of the course of infection.

If during imaging no signal is present (when it is known that the model should have an infection), there are a number of steps that can be followed to determine the source of the problem. Firstly, the exposure time can be increased to a maximum of 5 minutes which should be able to detect very low bioluminescence signal. If signal is still not detected and blood films are positive for parasite, the next step would be to confirm whether the parasite is still bioluminescent. This can be carried out by using a commercial *in vitro* luciferase activity assay and bioluminescence would be detected using a luminometer. If at this point the parasite is no longer bioluminescent, the stability of the construct in the parasite would need to be addressed.

The color of the mice makes a difference to the sensitivity of imaging as the emitted bioluminescence is attenuated by dark fur and skin[6,9]. Choice of mouse strain, such as white mice or nude mice, can therefore improve results. If mouse color is constrained, for example by use of a transgenic line, then shaving or depilating the mice in a region of interest can greatly improve sensitivity[6].

Despite the improved sensitivity and signal that bioluminescent model produces, the bioluminescence does not directly correlate to the peripheral parasitemia observed in blood films (days 7-21 shown in Figure 4). Literature has documented that the early stage infection of trypanosomiasis infects the hemolymphatic system[1], and is not solely restricted to the blood system and this, along with the infection of peripheral organs, may explain why when peripheral parasitemia is barely detectable in the blood films, bioluminescence is visible as is particularly apparent from the data for day 7 in Figure 3 and 4. This, however, does mean

that bioluminescence imaging can only be used as a qualitative measurement of infection, and further analysis is required to determine absolute parasite burden.

Bioluminescence imaging has the capability of being used with other infectious pathogens that are difficult to monitor, with rodent models of Chagas disease, malaria and toxoplasmosis already established[13-16]. The high signal-to-noise ratio of bioluminescence coupled with its ability to track real-time infections can provide a more sensitive, non-invasive drug assessment, as well as gain a better understanding of CNS infection kinetics, making it a valuable tool in infectious disease research.

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DISCLOSURES:
The authors have nothing to disclose.

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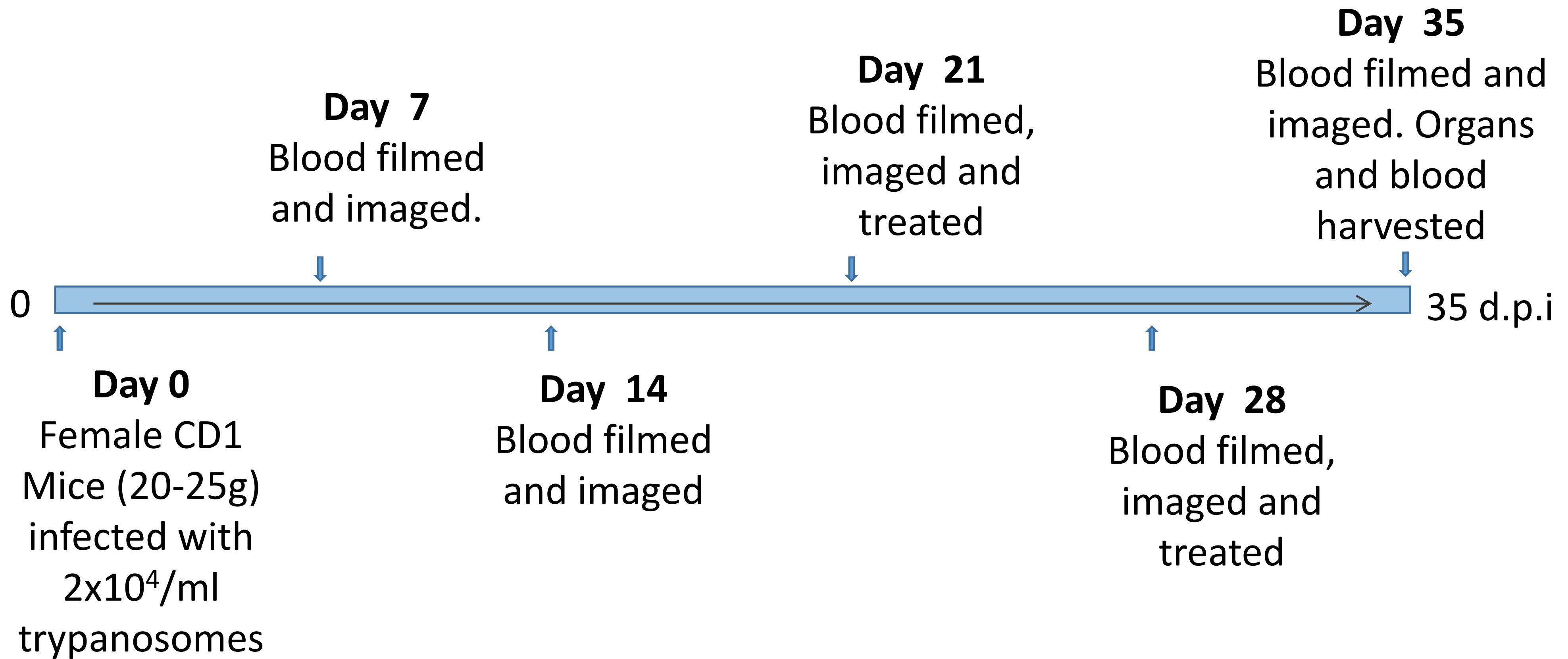
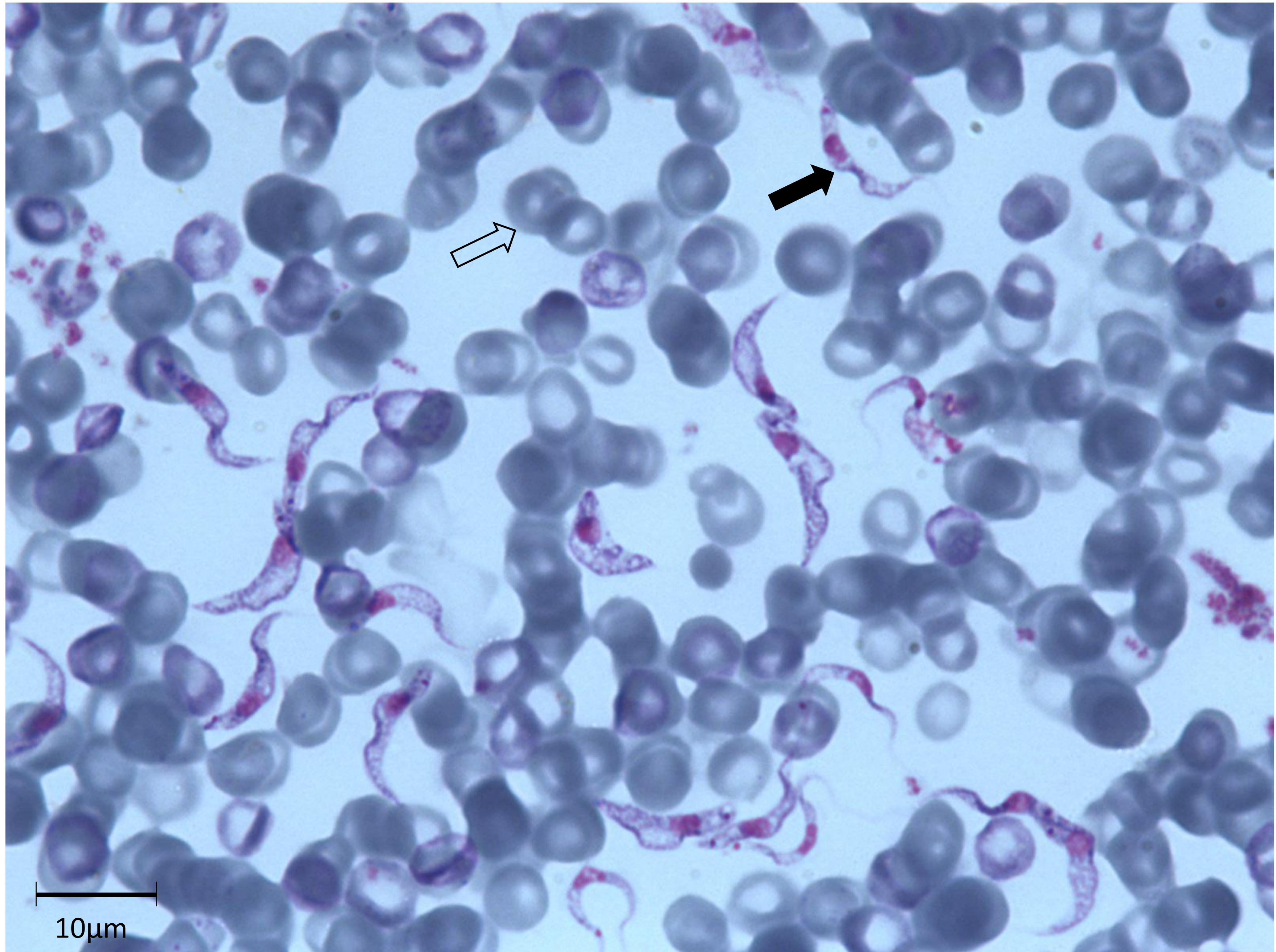

Day 35
Blood filmed and imaged. Organs and blood harvested
Day 21
Blood filmed, imaged and treated
Day 7
Blood filmed and imaged.
0
35 d.p.i
Day 0
Female CD1 Mice (20-25g) infected with $2x10^4$/ml trypanosomes
Day 14
Blood filmed and imaged
Day 28
Blood filmed, imaged and treated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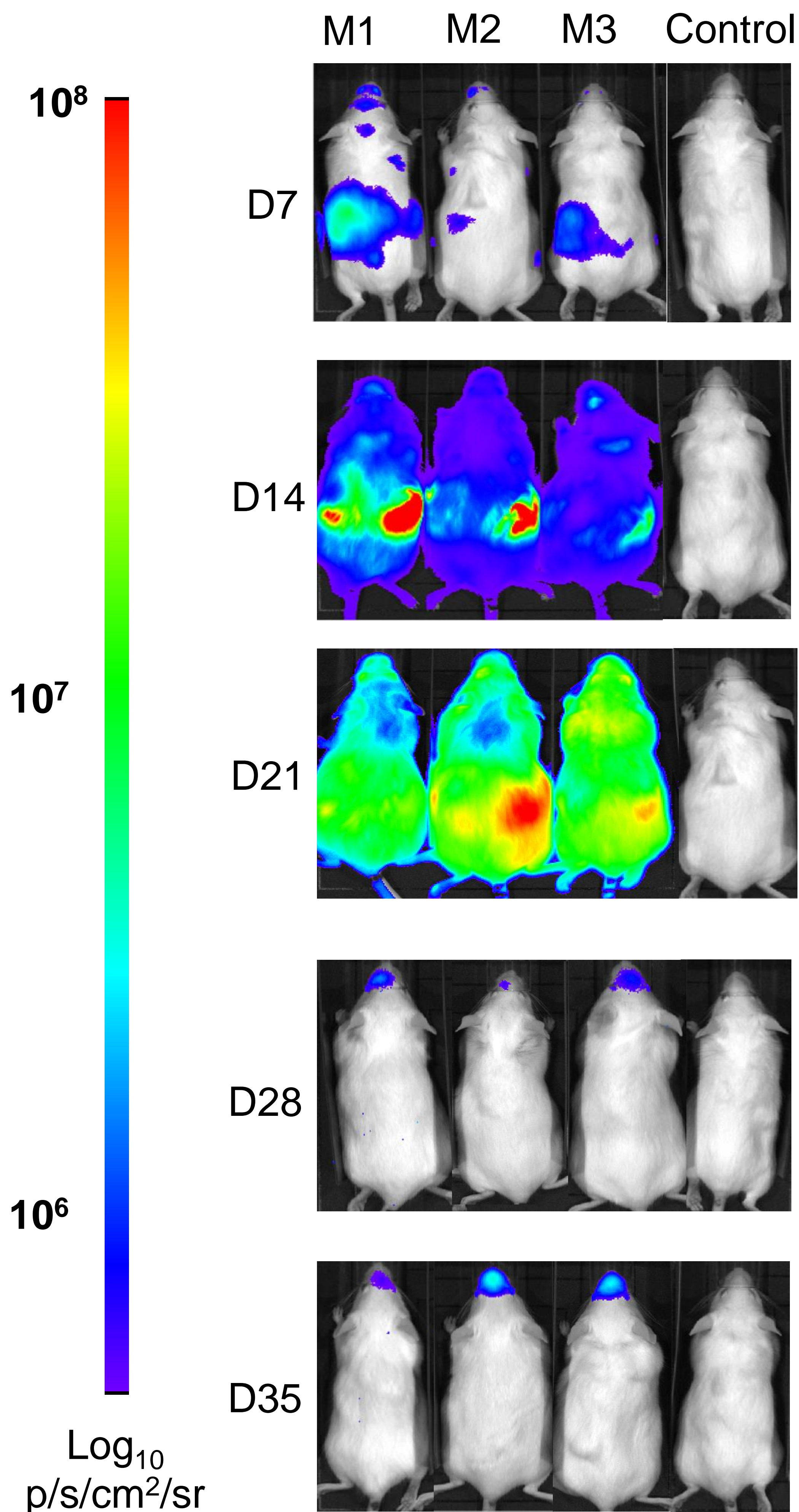
M1
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10^8
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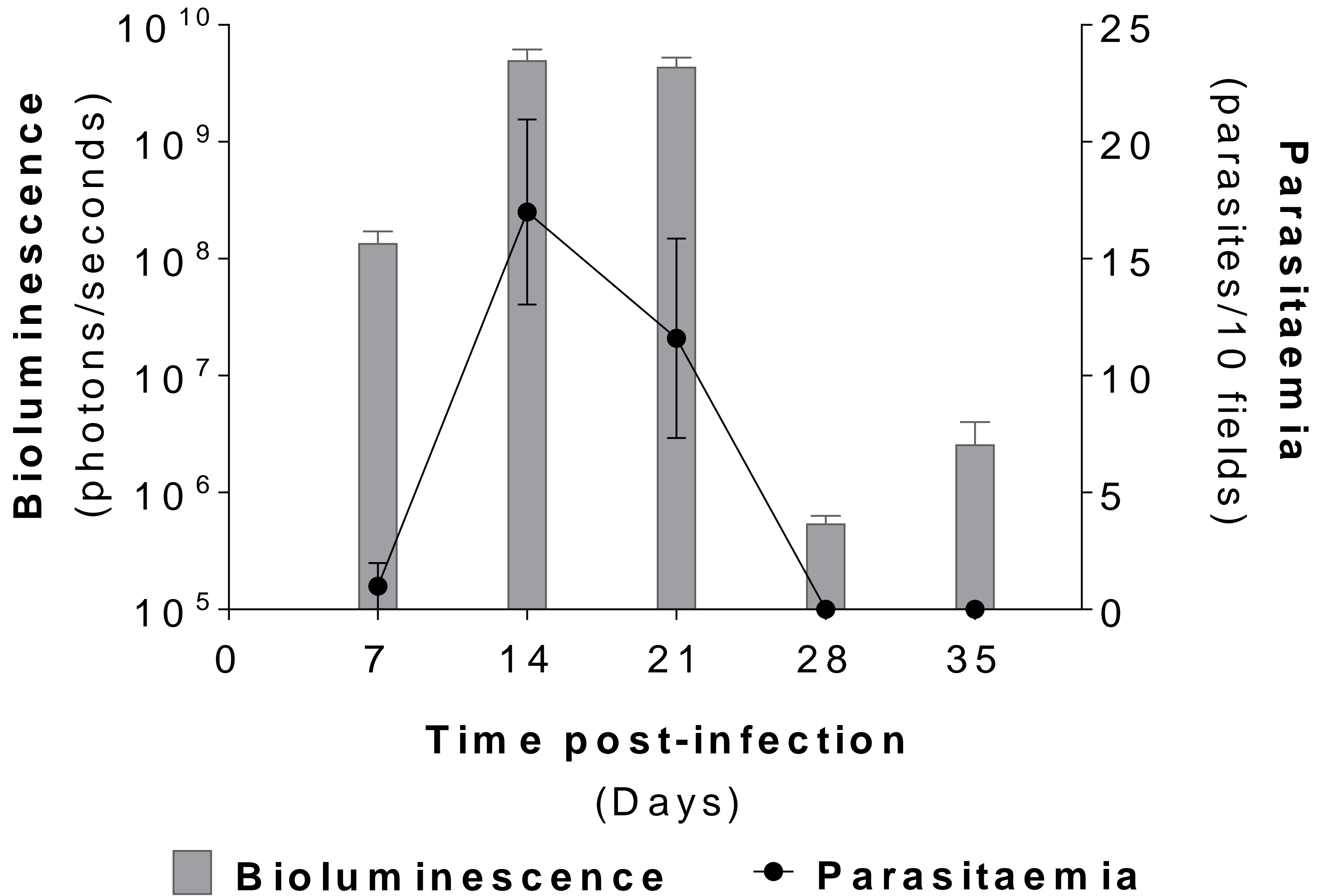
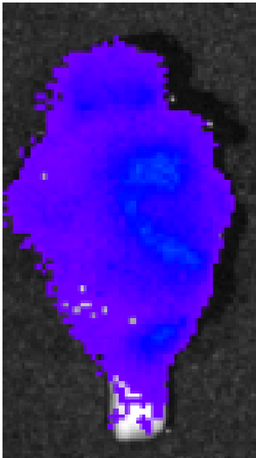
p/s/cm^2/sr

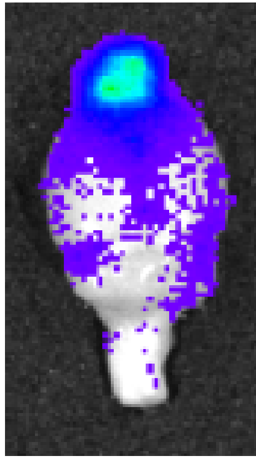


Bioluminescence (photons/seconds)
10^10
10^9
10^8
10^7
10^6
10^5
Parasitaemia (parasites/10 fields)
25
20
15
10
5
0
0
7
14
21
28
35
Time post-infection (Days)
Bioluminescence
Parasitaemia

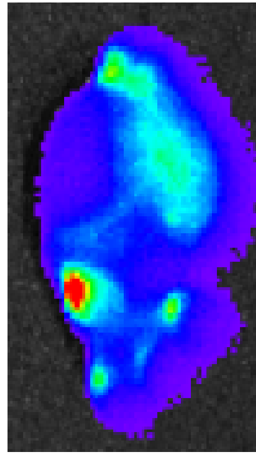
Mouse 1



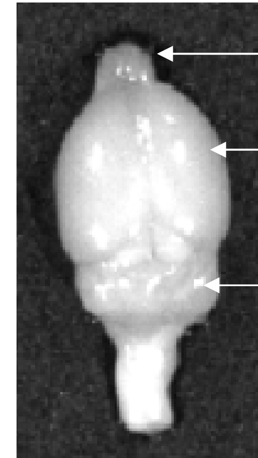
Mouse 2

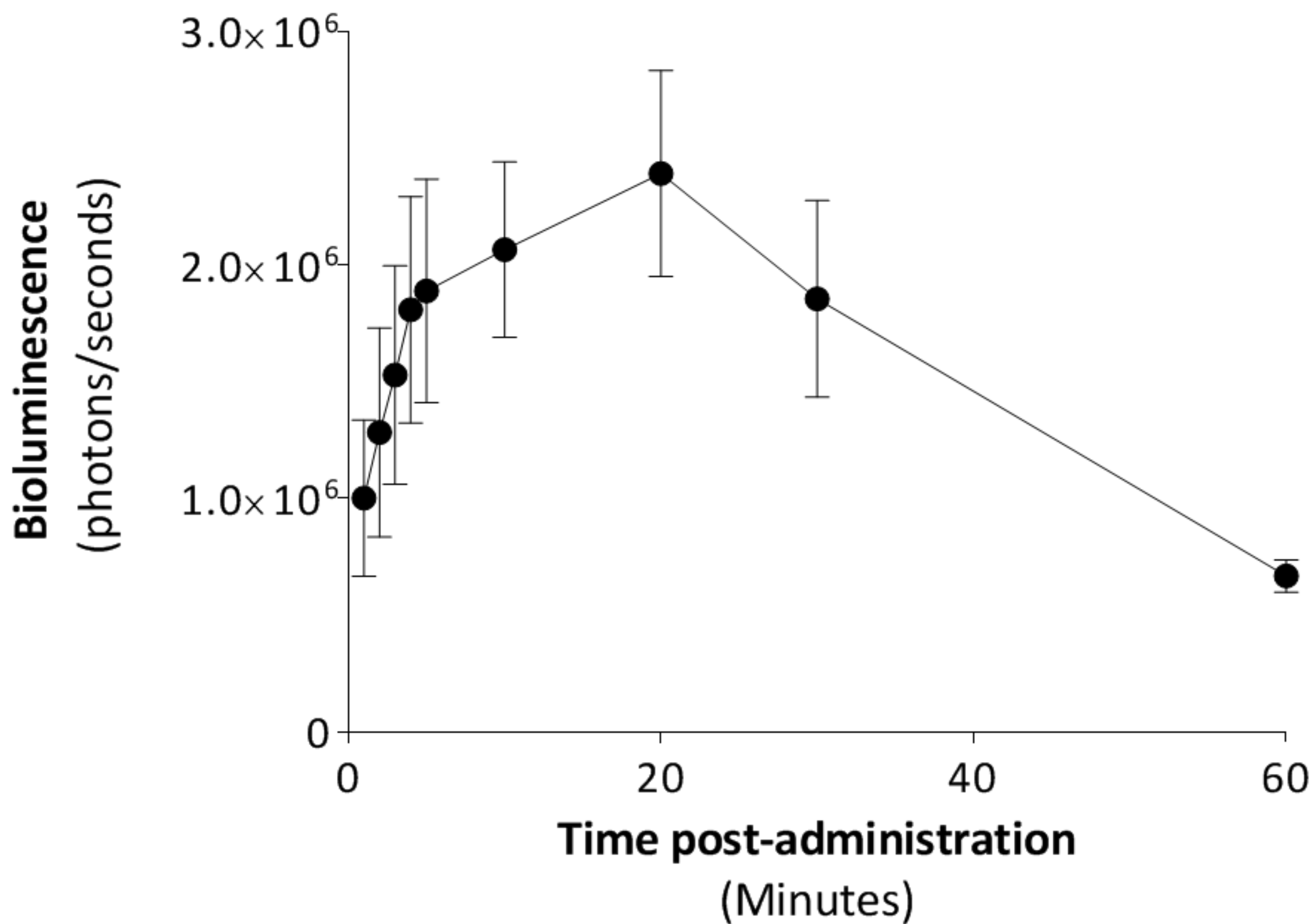


Mouse 3



Control




3.0×10^6
2.0×10^6
1.0×10^6
0
Bioluminescence
(photons/seconds)
0
20
40
60
Time post-administration
(Minutes)

Name of Material /Equipment	Company	Catalogue Number	Comments/Description
PBS	Sigma, UK	P4417	tablets pH 7.4
Glucose	Sigma, UK	G8270	99.5% (molecular) grade
Ammonium chloride	Sigma, UK	A9434	99.5% (molecular) grade
Heparin (lithium salt)	Sigma, UK	H0878	
Hi-FCS	Gibco, Life Technologies, UK	10500-064	500 ml
DPBS	Sigma, UK	D4031	Sterile filtered
Mr. Frosty	Nalgene, UK		
Giemsa	Sigma, UK	G5637	
D-Luciferin	Perkin Elmer, UK		
	Sigma, UK	115144-35-9	
Diminazene aceturate	Sigma, UK	D7770	Analytical grade
IVIS Lumina II	Perkin Elmer, UK		other bioimagers available e.g. from Bruker, Kodak
Living Image v. 4.2	Perkin Elmer, UK		proprietary software for Perkin Elmer IVIS instruments; other instruments may have their own
1 ml syringe	Fisher Scientific, UK	10142104	
20 ml syringe	Fisher Scientific, UK	10743785	
25G Needles	Greiner Bio-one	N2516	
21G Needles	Greiner Bio-one	N2138	
Twin-frosted microscope slide	VWR, UK	631-0117	
1.5 ml microcentrifuge tube	StarLab, UK	I1415-1000	
7 ml Bijou tube	StarLab, UK	E1412-0710	
Mouse restrainer	Sigma, UK	Z756903	our restrainer was made in-house, this is a similar model