Real-time *in vivo* imaging of transgenic bioluminescent blood stages of rodent malaria parasites in mice

Blandine Franke-Fayard, Andrew P Waters & Chris J Janse

Malaria Research Group, Department of Parasitology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. Correspondence should be addressed to C.J.J. (c.j.janse@lumc.nl).

Published online 27 June 2006; doi:10.1038/nprot.2006.69

This protocol describes a methodology for imaging the sequestration of infected erythrocytes of the rodent malaria parasite *Plasmodium berghei* in the bodies of live mice or in dissected organs, using a transgenic parasite that expresses luciferase. Real-time imaging of infected erythrocytes is performed by measuring bioluminescence produced by the enzymatic reaction between luciferase and its substrate luciferin, which is injected into the mice several minutes prior to imaging. The bioluminescence signal is detected by an intensified charge-coupled device (I-CCD) photon-counting video camera. Sequestration of infected erythrocytes is imaged during short-term infections with synchronous parasite development or during ongoing infections. With this technology, sequestration patterns of the schizont stage can be quantitatively analyzed within 1–2 d after infection. Real-time *in vivo* imaging of infected erythrocytes will provide increased insights into the dynamics of sequestration and its role in pathology, and can be used to evaluate strategies that prevent sequestration.

INTRODUCTION

Recent advances in genetic modification of malaria parasites and imaging technologies for visualizing cells expressing reporter genes have significantly broadened the possibilities for *in vivo* studies of interactions of malaria parasites with their hosts. To date, imaging of transgenic fluorescent or bioluminescent *Plasmodium* parasites has already provided exciting insights into the infection process[1,2].

In this protocol, methodologies are described for the real-time imaging of interactions of the blood stages of malaria parasites within whole bodies of live animals or isolated non-fixed organs[3]. Erythrocytes infected with the schizont stage of many species of malaria parasites adhere to endothelial cells of the microvasculature of numerous deep tissues[4,5]. This process of sequestration is mediated by parasite proteins expressed on the surface of the infected erythrocytes, which act as ligands for binding to receptor proteins expressed on the surface of host endothelial cells. In some parasite–host combinations, sequestration is associated with pathogenesis (e.g., *Plasmodium falciparum* in humans)[4–6]. Also, in (small) animal models of malaria, sequestration of schizonts occurs in the vasculature of inner organs and might cause pathology. Infection with *P. berghei* in laboratory rodents is a well-established model in which to study associations between pathology, pro-inflammatory cytokines and endothelial receptors involved in sequestration of infected erythrocytes, leucocytes and platelets[4,7]. However, studying the processes mediating sequestration in organs of humans or animals is difficult or ethically impossible, and most studies rely on post-mortem analyses or *in vitro* observations of infected erythrocyte–receptor interactions with cultured parasites and immobilized receptors[5].

This protocol describes three variations of imaging of sequestered schizonts of the rodent malarial parasite *P. berghei*. Methodologies are described for imaging both sequestered infected erythrocytes in whole bodies of mice with short-term synchronized infections of transgenic parasites, and sequestered transgenic schizonts in mice with ongoing infections of wild-type parasites, permitting the analysis of the dynamics of sequestration in a more established (ongoing) infection. In addition to whole-body imaging, we describe the imaging of the sequestration of transgenic schizonts in isolated non-fixed organs. All three methods are based on the published technology of Franke-Fayard *et al.*[3]. For real-time imaging of sequestered schizonts, a transgenic parasite is used that expresses the bioluminescent reporter protein luciferase. Imaging is performed by measuring the bioluminescence produced by the enzymatic reaction in the schizonts between the luciferase enzyme and its substrate luciferin, which is injected into the mice several minutes prior to imaging. The bioluminescence signal is detected by a sensitive intensified charge-coupled device (I-CCD) photon-counting video camera of the *in vivo*-imaging system (IVIS100) from Xenogen.

In the transgenic parasite, the reporter gene luciferase is under the control of the schizont-specific promoter *ama1*, resulting in expression of luciferase in schizonts. Expression is not only confined to the schizont stage, but young ring forms also contain luciferase as a result of carry over of the protein from the schizont stage to the newly infected erythrocyte. The development of the blood stages during 'natural' infections of *P. berghei* is not synchronous and bioluminescent ring forms can be present at the same time as the schizonts, which might hamper the specific imaging of the sequestering schizonts. Therefore, imaging is performed in experimentally induced synchronous infections in mice. In these infections, which are established by injection of purified mature schizonts[8], synchronicity of parasite development is maintained for two or three asexual cycles. The protocol therefore requires the generation of 0.5 to 1×10^8 cultured and purified schizonts to start synchronous infections in four mice. The collection of these schizonts is not described in this protocol, but is performed as described in the protocol detailing transfection of the schizont stage of *P. berghei* (Steps 1–22)[9].

In the synchronous infections, the optimal time to image sequestered schizonts is between 20 and 22 h after injection of the purified schizonts. Between 0 and 4 h after injection, schizonts rupture and the released merozoites will invade new erythrocytes. These parasites (i.e., ring-form stage) develop within 16–18 h into mature trophozoites (G1 phase of the cell cycle), which circulate in the peripheral blood circulation without sequestration. Eighteen hours after invasion, parasites enter mitosis (S/M phase; schizogony) and three rounds of mitotic division within 4–6 h result in the production of 12–16 merozoites. During the S/M phase (between 18 and 24 h after merozoite invasion), the infected erythrocytes (schizonts) disappear from the blood circulation as a result of their adherence to endothelial cells of blood capillaries of inner organs, such as lungs, spleen and adipose tissue. When the mature schizonts rupture, the merozoites invade new erythrocytes and start the next developmental cycle. As young ring forms (until 4 h after invasion) still express luciferase, it is important that the sequestration is imaged before the start of the new cycle of reinvasion.

It is well known that during the course of an infection, the expression of both host-cell receptors and parasite ligands involved in sequestration can change, which influences the pattern of sequestration[10,11]. To analyze such changes, a method is described for imaging sequestration of schizonts during the course of an infection. The 'artificially' induced synchronous infections of the transgenic parasites become asynchronous after only three asexual cycles, and this asynchronicity affects the imaging of schizonts because of the (background) luminescence signal in the whole body of the non-sequestering luminescent ring forms. To analyze schizont sequestration in ongoing infections, we therefore initially establish infections in mice with the (non-luminescent) wild-type parasite. At defined time points during these wild-type infections, sequestration of schizonts is imaged by injecting purified mature schizonts of the transgenic parasite.

The bioluminescence images of whole bodies of mice or isolated organs provide a qualitative assessment of the spatial distribution of sequestered schizonts within an animal, and this distribution can be directly compared between different animals if the same measurement settings during the experiments are maintained. Often, however, a quantitative analysis of the intensity of the bioluminescence signal is required, for example, to show variation in intensity between different animals or organs, or to demonstrate variations in intensity over time within the same animal. Using the Living Image software, quantitative data on the intensity of bioluminescence signals can be obtained by choosing the 'region of interest' (ROI) in the image for which the total integrated bioluminescence signal intensity is calculated. In this way, the bioluminescence intensity of complete animals or organs from different animals can be compared quantitatively.

Real-time *in vivo* imaging of sequestration of schizonts has already provided novel insights into the interactions of *P. berghei* and its host, as it revealed an unexpected tissue (adipose) in which sequestration occurs, and identified the main host receptor involved in schizont sequestration, the highly conserved class II scavenger receptor CD36, which is also the main receptor for erythrocytes infected with the human malaria parasite *P. falciparum*. It is anticipated that the use of this technology will provide increased insight into the dynamics of sequestration *in vivo* and the role of sequestration in pathology; in addition, this technology can be used to evaluate strategies to prevent sequestration and associated pathology.

MATERIALS

REAGENTS

- Parasite lines (see REAGENT SETUP)
- Parasite stages (see REAGENT SETUP)
- Mice (see REAGENT SETUP)
- Giemsa solution (Merck; cat. no. 1666 789): for a working solution dissolve 10% (vol/vol) Giemsa solution in Sorensen staining buffer
- MicroFine+ insulin syringes: 0.5 ml, 0.30 mm (30 G) × 8 mm (Becton Dickinson, cat. no. 324870)
- Artagain paper (Xenogen; cat. no. XBP-24)
- PBS (Roche; cat. no. 1.09204.0500) stock solution (10×): 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl; pH 7.0
- Sorensen staining buffer
- D-luciferin sodium salt (Synchem OHG; cat. no. BC218)

EQUIPMENT

- Biohazard class II safety cabinet **! CAUTION** Most manipulations with blood infected with genetically modified *P. berghei* parasites are performed in a class II safety cabinet
- IR heat lamp (home made or from Science Products)
- Carl Zeiss Standard 25 light microscope (Zeiss): all light microscopes with an 100× oil-immersed objective are suitable for this purpose
- Contura HS-40 shaver (Wella): used for removal of the fur from the skin of mice by shaving
- IVIS100 *in vivo*-imaging system (Xenogen)
- IGOR Pro 5 software (http://www.wavemetrics.com)
- XGI-8 gas-anesthesia system (Xenogen)

REAGENT SETUP

Parasite lines *P. berghei*, clone 15cy1 of the ANKA strain is used as a reference 'wild-type' line. *P. berghei*, mutant clone 354cl4 ($PbGFP–LUC_{SCH}$)[3] is a transgenic parasite line that expresses the fusion protein GFP–luciferase. The *gfp–luciferase* gene is under control of the schizont-specific promoter of the *ama1* gene of *P. berghei*. This line is available on request from the Leiden Malaria Research Group (http://www.lumc.nl/1040/research/malaria/malaria.html).

Parasite stages Schizonts of parasite 354cl4. The protocol requires the generation of 0.5 to 1×10^8 cultured and purified schizonts to establish a synchronous infection in four mice. These schizonts are obtained as described in the protocol detailing transfection of the schizont stage of *P. berghei* (Steps 1–22)[9].

Mice In our laboratory, mice of the following two strains are used: Swiss (OF1 ico, Construct 242, aged 6 wk, 25–26 g) and C57BL/6 (C57Bl/6Jico OF1, Construct 1, aged 6 wk, 20–25 g). The mice are obtained from Charles River. ▲ **CRITICAL** Diets of laboratory rodents with low contents of total protein, energy and *p*-aminobenzoic acid (PABA) can negatively influence *P. berghei* infections[12]. In our laboratory, we therefore provide diets with high protein (20–25% of total) and gross energy (18,000–20,000 kJ kg^{-1}) contents. **! CAUTION** All experiments using mice should be performed according to the applicable national guidelines and regulations.

PBS For a working solution, dilute the stock solution with nine volumes of distilled water. Adjust the pH to 7.2 with 1 M HCl and sterilize by autoclaving for 20 min at 120 °C.

Sorensen staining buffer For a working solution, dissolve 2.541 g KH_2PO_4 and 8.55 g $Na_2HPO_4 \cdot 2H_2O$ in 5 l distilled water, pH 7.2. Store at room temperature (19–23 °C).

D-luciferin sodium salt Dissolve 1 g D-luciferin in 12.5 ml PBS to give a stock solution of 80 mg ml^{-1} and store at –20 °C in the dark in 500-µl aliquots. Thaw the stock solution prior to use and inject into a mouse at a concentration of 120 mg kg^{-1} body weight (i.e., 30 µl for a mouse of 20 g).

EQUIPMENT SETUP

XGI-8 anesthesia system Used for anesthesia of mice prior to and during *in vivo* imaging. Mice are anesthetized in the 'induction chamber' which is pre-filled with the anesthetic vapor (isofluorane/oxygen) via the vaporizer unit, and are kept under anesthesia in the imaging chamber by holding their muzzles close to a small mask connected to the main vaporizer unit.

PROCEDURE

Infection of four mice (day 1, 12:00)

1| Suspend 0.5 to 1 × 10^8 cultured and purified schizonts in 800 µl of complete culture medium in a biohazard class II safety cabinet (with care because the schizonts are fragile). These schizonts are obtained as described in the protocol detailing transfection of the schizont stage of *P. berghei* [9].

2| With an insulin syringe, inject 200 µl of the schizont suspension into a tail vein of each of the four mice under anesthesia. The injection of mice with schizonts should be performed at 12:00. This time point corresponds to $t = 0$ of the synchronous infection (see below). If you wish to image sequestered schizonts of transgenic parasites during ongoing infections in mice with wild-type *P. berghei*, then inject the schizont suspension into four mice with ongoing infections of wild-type parasites. The method of establishing wild-type infections in mice is described in **Box 1**.

▲ **CRITICAL STEP** 5–10 min before injection of the parasites, place the mice under an IR heat lamp. The tail veins swell at the higher temperature, simplifying the intravenous-injection procedure. In addition, prepare the XGI-8 gas-anesthesia system in sufficient time, such as filling the 'induction chamber' with the anesthetic vapor (isofluorane/oxygen), to be able to inject the schizonts immediately after the purification procedure.

Preparing thin blood smears (day 1, 16:00)

3| Make a thin blood smear on a microscope slide from one droplet of tail blood from each of the four infected mice at 16:00 ($t = 4$).

4| Fix the smears for 2 s with methanol and stain with a fresh Giemsa working solution for 10 min.

5| Wash the slide with tap water.

6| Air dry the slide and determine the parasitemia using a light microscope (with immersion oil and objective at 100×) by counting 10–20 fields of 300–400 erythrocytes per field.

7| If the parasitemia ranges between 0.5 and 3% proceed with Step 8 (if not, see below).

▲ **CRITICAL STEP** Injection of the schizonts results in rapid invasion of new erythrocytes within 2–4 h after injection. Usually, the parasitemia increases during this period to levels between 0.5 and 3%. It is difficult to adjust the number of injected schizonts in such a way that mice will develop an infection with precisely defined parasitemia. The exact parasitemia is dependent on multiple factors, such as the maturity/viability of the cultured schizonts, the success of the injection procedure and the reticulocyte percentage in the blood of the mouse. It is recommended to aim for a parasitemia between 0.5 and 3%. Lower parasitemia might

BOX 1 ESTABLISHMENT OF ONGOING INFECTIONS WITH WILD-TYPE PARASITES

To analyze sequestration of schizonts (parasite 354cl4) during an ongoing infection, mice are first inoculated with wild-type *P. berghei*. After the wild-type infection has been established, these mice are injected with purified schizonts of parasite 354cl4 as described in Steps 1 and 2. Injection of these schizonts is performed at day 5 or 6 after the start of the wild-type infections.

(1) Collect one to three droplets (4–12 µl) of tail blood in 8 ml PBS from a mouse infected with wild-type parasites of *P. berghei* (ANKA strain, clone 15cy1). The parasitemia (i.e., the percentage of infected red blood cells) in this mouse must be in the range of 5 to 15%.
(2) Immediately inject 5 × 10^4 infected erythrocytes (~0.1 ml of the cell suspension per mouse) via the i.p. route into four naive mice.
(3) At day 5 or 6, inject these mice with purified schizonts of parasite 354cl4, as described in Steps 1 and 2, and image schizont sequestration at day 6/7 and 7/8, as described in Steps 8–26. Note that the infected mice will develop an infection with wild-type parasites in which the parasitemia increases from 0.01–0.1% at day 4 after infection to levels of 10–15% at day 6 or 7. In these infections, clinical symptoms of malaria, such as cerebral complications, become evident at day 7 or 8. To image schizont sequestration, these mice are injected with purified schizonts from the transgenic parasite 354cl4 at day 5 or 6 of the wild-type infections. Imaging at day 6 or 7 (and possibly day 8) will therefore demonstrate sequestration patterns at a time point just before manifestation of the clinical symptoms.

limit the collection of bioluminescence data because of the current sensitivity limits of the method for detecting sequestered schizonts in all organs. A higher parasitemia results in less-synchronous development of the parasites resulting from the presence of multiple-infected erythrocytes in which development of the parasites is impaired.
? TROUBLESHOOTING

Imaging of sequestered schizonts (day 1, 23:00)

8| Imaging can be performed on whole bodies of mice at various time points (A) or on dissected individual organs of mice (B). Option B should be used if a more detailed image of sequestration in individual organs is required than can be obtained from imaging whole bodies of mice. Imaging of isolated organs can provide additional data on the precise localization of the signals, and can result in a higher imaging sensitivity by preventing a 'quenching effect' from other tissues.

(A) Imaging of whole bodies of mice.

(i) 23:00 is the first time point at which the bioluminescence signal of the infected mice is measured (Steps 9–25). This time point, $t = 11$, is used as a 'negative control' for the bioluminescence signal, because no expression of luciferase takes place. At earlier time points, between $t = 0$ and $t = 8$, some background bioluminescence might be measured as the result of the presence of residual schizonts (from injection) and young ring forms. With the infected mouse under anesthesia, remove the fur from the ventral part of the body by shaving using a Contura HS-40 shaver. Removal of the fur is performed to prevent quenching of the light signal, and must be done carefully as a hematoma might influence the imaging.

(B) Imaging of dissected isolated organs.

(i) Organs can be obtained from mice with a synchronized 354cl4 infection or an ongoing infection of wild-type parasites with sequestered schizonts of 354cl4.

9| Prepare the IVIS100 system (**Fig. 1**) for imaging the mice or organs at $t = 11$.
▲ CRITICAL STEP Use the IVIS100 system as recommended by the manufacturer. Ensure that the IVIS is operational and that the automatic background measurements have been performed with settings that will be used for imaging the mice (see below). The Xenogen custom Living Image software runs the IVIS100 system. This software automates many settings of important functions, such as background management, and data storage and retrieval. The software also serves as a guide to help (inexperienced) users through the steps associated with quantitative *in vivo* imaging and data analysis.

10| Start the Living Image software program on the screen from the Windows start menu, register your identification (ID) and click 'Done'.

11| Click the 'Initialize IVIS system' button in the 'Camera control' panel. This will move every motor-driven component of the imaging system (e.g., sample stage and lens; **Fig. 1**) to a 'home' position, reset all electronics and controllers, and restore all software variables to default settings.

12| Adjust the field of view (FOV) of the 'Camera control' panel to A (10 cm). This control sets the FOV by adjusting the distance between the camera and the sample stage (**Fig. 1**). FOV is set to provide an imaging area that is wide enough to encompass the entire sample or the area of interest. Distances of 10 cm (A) are used for imaging a whole body of a mouse or isolated organs in a Petri dish.

13| Usually, the default settings of the 'Camera control' panel are used to record the bioluminescence signal (with an exposure time of 60 s and parameters such as 'Binning' and 'f/stop/Focus'). The bioluminescence signal collected is linearly related to the exposure time within a range of 5 s to 10 min. If the signal intensity of the bioluminescence signal requires a longer or shorter exposure time, make sure that the new exposure time is also listed in the 'LI auto background list' (see Step 9, **▲ CRITICAL STEP**).

14| Switch on the XGI-8 anesthesia system that is connected to the IVIS100 to pre-fill the induction chamber (**Fig. 1**) with isofluorane and oxygen.

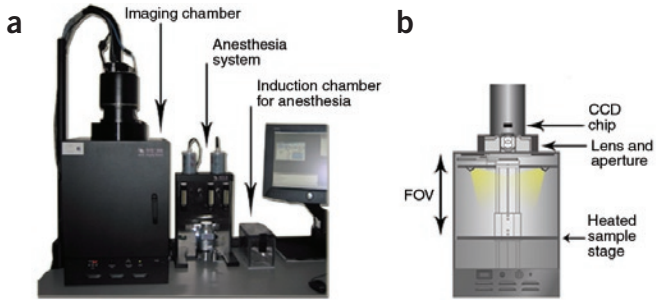

Figure 1 | *In vivo* imaging system, IVIS100 from Xenogen, used for imaging luciferase-expressing sequestered schizonts in whole bodies of live mice or in isolated non-fixed organs. Imaging of the parasites is performed by measuring bioluminescence (photons) produced by the enzymatic reaction between the luciferase enzyme expressed in the schizont stage and its substrate luciferin injected into the mice, several minutes prior to imaging (Source: Xenogen IVIS 100 with Living Image software). (**a**) The mice are anesthetized in the induction chamber of the Xenogen XGI-8 gas-anesthesia system and are kept under anesthesia during imaging in the imaging chamber. (**b**) The bioluminescence signal is detected by a sensitive I-CCD photon-counting video camera. The FOV is defined by the distance between the camera and the sample stage on which the mice or organs are placed.

15| Place one mouse in the induction chamber to be anaesthetized.
▲ **CRITICAL STEP** The Xenogen XGI-8 gas-anesthesia system is designed to operate with the IVIS100 imaging system. Gas anesthesia is recommended above anesthesia induced by i.p. injection of chemicals, because its depth and duration can be more easily controlled and standardized. It is important that the mice recover quickly from anesthesia after the measurements, as anesthetized mice show a drop in body temperature that affects the growth of the parasite. Therefore, during prolonged or repeated anesthesia, mice should be kept at 37 °C.

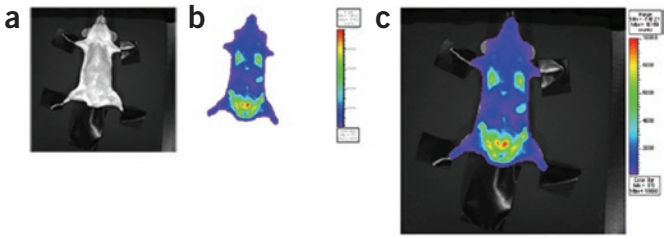

Figure 2 | Imaging of luciferase-expressing schizonts in a live mouse (**a**) Photographic image of a mouse obtained with the IVIS100 system. (**b**) Luminescent image of the same mouse. Rainbow images show the relative levels of luciferase activity, ranging from low (blue), to medium (green), to high (yellow, red). (**c**) The 'overlay' of the photographic and luminescent images.

16| Inject 30 µl D-luciferin substrate solution subcutaneously into the neck of the anesthetized mouse.
▲ **CRITICAL STEP** Reproducible imaging results are obtained with subcutaneous injection of the substrate. However, we have evidence that intravenous injection of D-luciferin might improve the sensitivity of imaging, especially for organs that could degrade/eliminate the substrate more rapidly or are less accessible for the substrate (e.g., the liver).

17| If you wish to image the whole body of the mouse, follow option (A). If you wish to dissect the organs, follow option (B).
(A) Imaging of the whole body.
(i) Place the mouse on a piece of Artagain paper, fix it by taping the legs, and position it under the camera in the centre of the sample stage. Close the door of the imaging chamber. The gated sample stage is pre-warmed to 37 °C and thereby stabilizes the body temperature of the mouse.
(ii) Wait for 3 min before acquiring the bioluminescence image. This period allows circulation of the D-luciferin substrate within the body of the mouse. Proceed with Step 18.
▲ **CRITICAL STEP** Remove all dust particles from the sample stage, and use only black paper and tape for fixing the mice to prevent background light emission.
(B) Imaging of dissected organs.
(i) Wait for a period of 3 min before dissecting the organs. This period allows circulation of the D-luciferin substrate within the body of the mouse.
(ii) Dissect the desired organs from the anesthetized mouse as quickly as possible and place them on the cover plate of a Petri dish.
(iii) Remove blood traces carefully and wet the organs with PBS to prevent dehydration.
(iv) Place the Petri dish with the organs on the sample stage of the IVIS100 system and leave the organs at 37 °C for 1 min in the imaging chamber prior to imaging (Step 18).
▲ **CRITICAL STEP** To facilitate the analysis of signal intensities in organs from different mice, it is recommended that they are placed in the same relative order in the Petri dish and in the same position in the imaging chamber. This allows the use of preset and saved ROI, as described in Steps 30–32. If bioluminescence signals of the various organs differ significantly in intensity, it is advisable to image organs separately (light quenching is higher in 'dark' and large organs). This allows low signals to be imaged by adjusting the exposure time per individual organ. For quantitative comparison of signal intensities of organs from different mice, it is best to use organs from those with comparable parasitemia.

18| Click on 'Acquire continuous photos' in the 'Camera control' panel, resulting in the display of a photographic image of the mouse or organs on the monitor (**Fig. 2**).

19| Check the correct position of the animal or organs. If the position is not correct, stop the recording of the image, adjust the position and resume imaging by clicking on 'Acquire continuous photos'.

20| Check the 'Overlay box' in the 'Camera control' panel and click 'Acquire', resulting in the acquisition of the luminescent image of the mouse or organs (**Fig. 2**).

21| After exposure is complete, the 'overlay' of the photographic and luminescent picture is displayed (**Fig. 2**). Enter the information in the 'Change information' window and click 'Done'.
▲ **CRITICAL STEP** The unit for the image-acquisition setup is 'Counts' (Xenogen recommendation).

22| In the 'Living image' menu, choose 'Save living image data' to save the displayed images and data

23| If necessary, acquire a new image (Steps 18–22) with shorter or longer exposure time (adjust the exposure time in the 'Camera control' panel in Step 18). ▲ **CRITICAL STEP** Confirm that the bioluminescence signal is above the noise level (recommended >100 counts) and below CCD saturation (65,535 counts). At high (>2%) or low (<0.5%) parasitemia, the default exposure time of 60 s might result in overexposed or underexposed images, respectively. If the exposure time is adjusted, make sure that the new value is also listed in the 'LI auto background list'.

24| Remove the mouse or organs from the imaging chamber and repeat Steps 15–23 for a new mouse or set of organs. If all images have been recorded, proceed with Step 25.
▲ **CRITICAL STEP** Recording imaging data with the IVIS100 system is usually performed for each mouse separately. It is possible to measure bioluminescence signals from up to five mice at the same time. However, if bioluminescence signals differ significantly in intensity between mice, it is more difficult to collect the images of the (individual) mice with the correct exposure time and to analyze the image data for quantitative comparisons (**Fig. 3**).

25| Close the program via the tool bar menu: File/Exit.

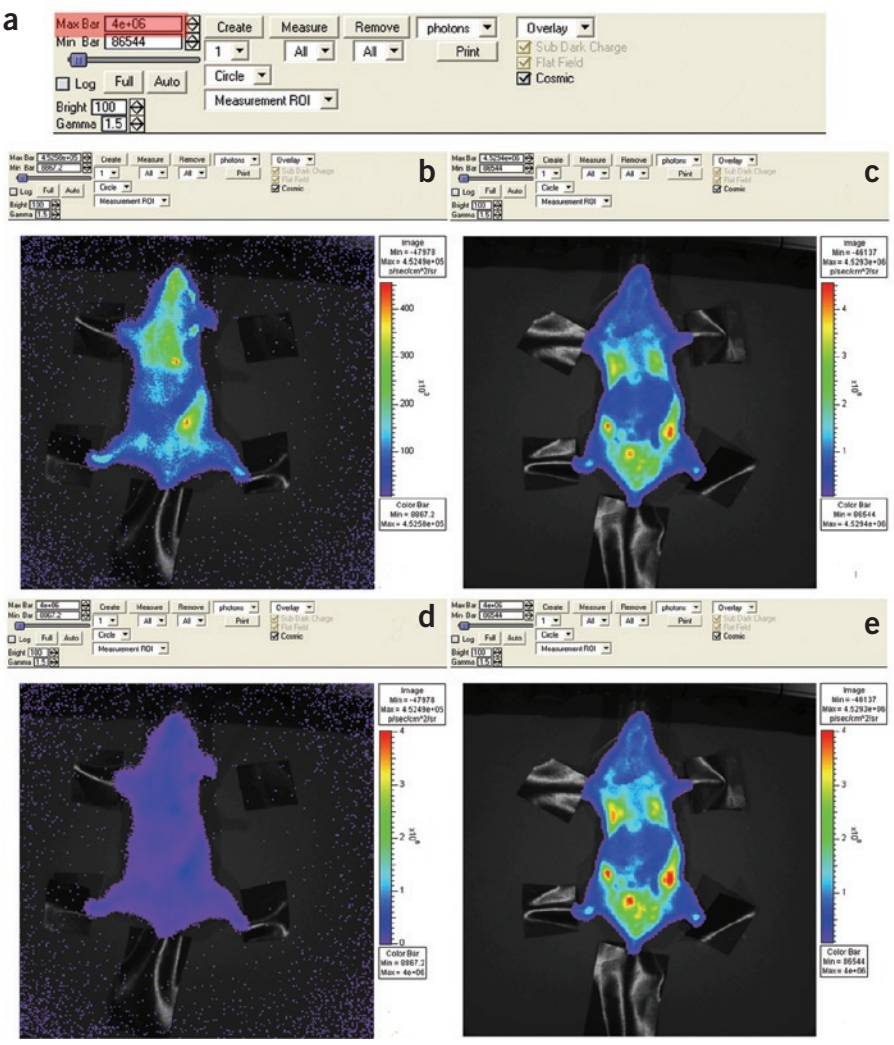

Figure 3 | Comparative analysis of bioluminescence intensities of two different mice. (**a**) The 'Controls section' of a 'Living image window' with the 'Max bar' that sets the maximum value of the scale of the 'Color bar' for the image highlighted in orange. (**b**–**e**) The pictures of the mice show the effect of the standardization of the 'Color bar' scale on images acquired with similar camera settings. Raw data of bioluminescence images of two different mice are shown in **b** and **c**. The same mice are shown in **d** and **e** after standardization of the bioluminescence scale bar by setting the 'Max bar' at 4×10^6 photons for both mice. For quantitative comparison of differences in signal intensities between mice, the same value of the 'Max bar' is chosen, whereas for detection and visualization of signals of low intensity in individual mice, different settings of this value can be used.

Day 2

26| Repeat Steps 8–25 at 8:00 (t = 20), 10:00 (t = 22) and 16:00 (t = 28) to record the bioluminescence images for all mice (**Fig. 4**).
▲ **CRITICAL STEP** When using mice with an ongoing wild-type infection, experiments are usually terminated after the last time point before clinical symptoms of cerebral complications become apparent.
■ **PAUSE POINT** Images can be stored for analysis when convenient.

Image analysis

27| Start the Living Image software.

28| Click on 'Living image' on the Tool bar menu, select 'Browse for LI data' (Image retrieval) and choose the directory in which the imaging data have been saved (Step 22). A 'Control panel' and 'Table' are displayed. The table displays the image data in the directory. Use the 'Control panel' to choose, delete, sort or load selected images, or to browse for more images. Each row represents an image. Each column represents the user information entered when the image was acquired (Steps 18–22), the instrument parameters for that acquisition, and a time and date stamp.

29| Choose the images to be viewed, highlight them using the 'Shift' key and click 'Load'. The information in the table can be sorted by column or by any combination using the 'Sort' button, allowing comparison of animals/organs that have been imaged within the same experimental setting (e.g., measured at the same time point) and/or with the same camera settings (e.g., FOV and/or time of exposure).

30| Create the ROI on the images to be analyzed (**Fig. 5**). The ROI can be the whole body, specific regions of the mice or specific organs. The ROI is created using 'Image window control–Create'. This allows more than one ROI to be defined. For quantification of the bioluminescence signal using ROI, it is advisable to use the units 'Photons' and not 'Counts' (see the user manual for the Living Image software from Xenogen for more details).

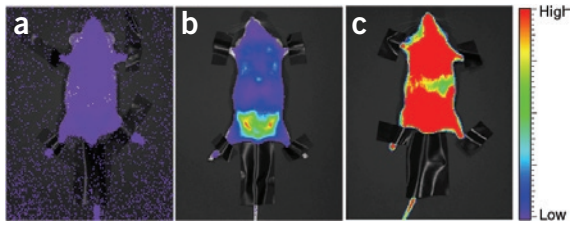

Figure 4 | *In vivo* imaging of sequestered schizonts in whole bodies of live mice with synchronized infections of the luciferase-expressing parasite line 354cl4. Images are acquired at different time points after injection of purified mature schizonts. (**a**) At t = 11, trophozoites are present in the blood that do not sequester or express luciferase, resulting in the absence of a bioluminescence signal. (**b**) At t = 20 and t = 22, luciferase-expressing schizonts are present that sequester in the lungs and adipose tissue, and accumulate in the spleen. (**c**) At t = 28, the mature schizonts have ruptured and luciferase-expressing ring forms are present in the peripheral blood circulation, resulting in a strong signal in the whole body of the mouse.

31| Save the settings of the ROI by clicking on the 'Save ROI' command in the 'Preferences' panel in 'Tools'. The settings of the ROI can be saved for use in subsequent analysis of other images.

32| Measure the intensity of the bioluminescence signal in the ROI. 'Image window control–Measure' initiates the measurement of the signals of individual or multiple ROIs present in the image. The measurement of the intensity of bioluminescence signals in the ROI results in the generation of a 'measurement table' that contains both the total and average number of counts for each ROI.

33| Save the data according to either option (A) or option (B).

(A) 'Living image–Save living image data'.

(i) This creates, for each file, a folder that contains image data in a generic form, and additional information, such as camera settings, in a text file. The data can be saved in either 'raw data' or 'analyzed data' format.

(B) 'File–Save experiment as'.

(i) This creates an 'IGOR Pro 5' software file that, when opened, reproduces all data present on the screen at the time of saving, such as annotations made on the images or analysis of ROI. The drawback is that this format is Igor Pro-software specific and cannot be used with other software.

▲ **CRITICAL STEP** For both options, it is advisable to keep an archive of the experimental raw data and to save the analyzed data separately. 'Living image–Load LI data' can be used to locate and load the specific raw or analyzed data sets.

? TROUBLESHOOTING

● **TIMING**

Imaging of sequestration of schizonts in synchronized short-term infections. Day 1: purification of schizonts and injection into four naive mice (2 h); preparing blood smears to check parasitemia (1 h); imaging of four mice at t = 11 (23:00; 1.5 h). Day 2: imaging of four mice at t = 20 – 22 (8:00 to 10:00; 1.5 h); optional dissection of organs and imaging of organs (2 h); imaging of four mice at t = 28 (16:00; 1.5 h). Final day: image analysis (2 h).

Imaging of sequestration of schizonts during ongoing infections. Day 1: Infection of four mice with wild-type parasites (1.5 h). Day 5 or 6: purification of schizonts and injection into the four mice infected at day 1 (2 h); preparing blood smears to check parasitemia (1 h); imaging of four mice

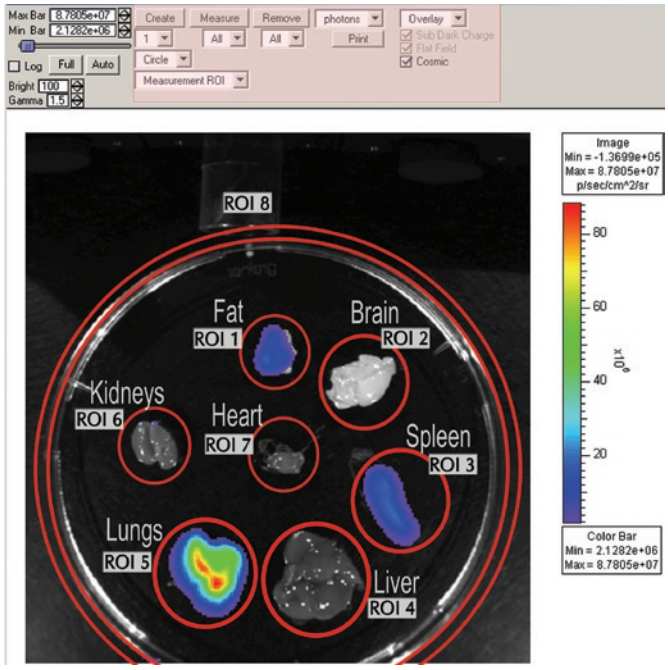

Figure 5 | Comparative analysis of bioluminescence signal intensities of isolated organs using the ROI option. The top panel shows the 'Controls section' of a 'Living image window' with, highlighted in orange, the commands of the ROI option (for an explanation, see main text). The lower panel shows the different, manually set ROI over the different organs.

at t = 11 (23:00; 1.5 h). Day 6 or 7: imaging of four mice at t = 20 – 22 (8:00 to 10:00; 1.5 h); optional dissection of organs and imaging of organs (2 h); imaging of four mice at t = 28 (16:00; 1.5 h). Final day: image analysis (2 h).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
7	The parasitemia is >3% at 4 h after injection of the schizonts.	Too many schizonts have been injected.	Adjust the dose of injected schizonts. At a parasitemia of >3%, parasite development is impaired as a result of multiple-infected cells, resulting in a less synchronous development of the parasites.
	The parasitemia is <0.5% at 4 h after injection of the schizonts.	Too few viable schizonts have been injected or the intravenous injection failed.	Adjust the dose of injected schizonts or improve the injection method. At a parasitemia of <0.5, less accurate imaging data will be obtained as a result of the sensitivity of measurement of bioluminescence. As synchronicity of development is maintained for at least two cycles, one might perform the imaging during the second cycle when the parasitemia is between 0.5 and 3%.
33	A low bioluminescence signal in lungs compared to belly fat, or a high signal in lungs compared to belly fat, at t = 20 – 22.	The relative intensity is dependent on the amount of belly fat. In addition, we have evidence that in certain animals, for example rats, schizonts seem to sequester preferentially in the fat and less in lung tissue.	The diet (food) of the mice influences the amount of belly fat. Because it is known that the protein content of the diet can influence parasite growth in mice, we provide a diet with relatively high protein content.
	A relatively strong bioluminescence signal in the whole body, especially the bare parts such as tail, nose and feet, at t = 20 – 22.	The luminescence imaging was performed at a time point that schizonts had ruptured, resulting in the presence of bioluminescent ring forms in the blood circulation.	Adjust the timing of the imaging, according to the developmental cycle of the parasites.
		The infection is not synchronous, resulting in the presence of luminescent ring forms in the circulation.	Make sure that only highly purified and mature schizonts are injected to start a synchronous infection.
		Schizonts do not sequester.	The level and exact localization of sequestration of *P. berghei* schizonts is dependent on both the parasite line and host (factors). Take care that the right parasite line and mouse strain are used for the studies.
	A relatively strong luminescent signal is observed at t = 11.	The infection is not synchronous, resulting in the presence of sequestering schizonts at t = 11 and/or luminescent ring forms in the circulation.	Make sure that only highly purified and mature schizonts are injected to start a synchronous infection.

ANTICIPATED RESULTS

The use of the methodology for *in vivo* imaging of transgenic luciferase-expressing malaria parasites will provide data on the temporal and spatial distribution of infected erythrocytes in live animals, and give insight into the dynamics of the sequestration characteristics of schizonts in tissues of different organs. A published study using this technology revealed a novel tissue for sequestration (i.e., adipose tissue) and demonstrated that the highly conserved class II scavenger receptor CD36 on endothelial cells of blood capillaries is the main receptor involved in the sequestration of *P. berghei* schizonts[3].

To date, most data on distribution patterns of sequestered malaria schizonts in the different organs have been obtained from post-mortem studies. Such studies cannot provide a complete picture of the dynamics of sequestration during the

course of an infection, or of the host and parasite factors affecting sequestration patterns. In addition, analysis of the characteristics of sequestered schizonts, such as the interactions of parasite adhesins with host-cell receptors, is mainly performed *in vitro* with cultured parasites and immobilized receptors[5]. It is, however, questionable as to what degree such interactions *in vitro* are representative of the *in vivo* interactions between parasite adhesins and host-cell receptors, which occur in the dynamic environment of blood vasculature of inner organs[13]. Therefore, the clear advantage of the methodology of *in vivo* imaging is that sequestration and its dynamics can be visualized and studied in the 'natural' environment of the living host. Moreover, bioluminescence imaging is simple to execute, allows monitoring of the course of biological processes without killing the experimental animal, and, therefore, can reduce the number of animals required for experimentation because multiple measurements can be made in the same animal over time, minimizing the effects of biological variation[14–16]. *In vivo* imaging, in combination with the use of both transgenic parasites that express modified ligands and transgenic mice expressing modified endothelial cell receptors, will allow detailed experimental approaches to investigate adhesion receptor interactions *in vivo*. For example, transgenic *P. berghei* parasites expressing known adhesion domains of *P. falciparum* (e.g., DBL or CIDR domains of PfEMP1 proteins) and transgenic mice with additional human endothelial cell receptors (e.g., ICAM-I (ref. 17)) might serve as small-animal models for the study of human parasite adhesins and human receptor interactions *in vivo,* and might be used for *in vivo* screening of inhibitors of *P. falciparum* sequestration. Moreover, in combination with knockout mice that are deficient in immune-response pathways, *in vivo* imaging of the parasites might help unravel the complexities of parasite–host interactions that lead to pathology.

It is clear that the methodology of *in vivo* imaging of sequestration at the level of entire bodies or in dissected organs of mice measures population effects, and will therefore mainly provide insight into factors that will affect more global changes in spatial and temporal sequestration patterns. The visualization of small changes in sequestration patterns or behavior of schizonts is dependent on the sensitivity of measuring the luciferase activity, the ability to reliably measure quantitative differences between different organs, and the resolution capability of the optical systems used for visualization. Both quantitative measurements of (low) signals and the exact localization of the signals is dependent on light-absorption (quenching) and light-scattering effects of the bioluminescence signal, which differ between the various organs. Therefore, although bioluminescence imaging provides a unique and powerful methodology, quantitative analysis must be approached with caution, and validation for each specific application is necessary[14]. In order to obtain a complete picture of the *in vivo*-sequestration processes in different organs, the relatively 'low-resolution' methodology of *in vivo* imaging of luminescent parasites needs to be combined with intravital-imaging technologies with cellular resolution, such as wide-field, spinning-disc or multiphoton-confocal microscopy[1,2,18]; the last of these combines the advanced optical techniques of laser-scanning microscopy with long-wavelength multiphoton-fluorescence excitation to capture high-resolution 3D images of living tissues that have been tagged with specific fluorophores. Advances in this technology[19], combined with parasites expressing fluorescent markers such as GFP or red fluorescent protein (RFP), now make it possible to observe parasite–host interactions at the level of individual cells in live animals[1,2].

Sequestration of *P. falciparum* schizonts is thought to play an important role in the induction of inflammatory processes that can lead to pathology. The exact (molecular) mechanisms of induction of inflammatory immune responses by sequestered schizonts remain, however, unknown. Infection with *P. berghei* in laboratory rodents is a widely used model for the investigation of associations between pathology, pro-inflammatory cytokines and endothelial receptors involved in sequestration of schizonts, leucocytes and platelets[7,20]. The combination of different methodologies for *in vivo* imaging of both parasites and specific host cells, such as specific leukocyte populations and platelets that play a role in sequestration, might help to unravel the complex interactions that lead to pathology.

ACKNOWLEDGMENTS We would like to thank H. Kroeze, J. Ramesar and S. Khan for their critical comments.

COMPETING INTERESTS STATEMENTS The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Rights and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Amino, R., Menard, R. & Frischknecht, F. *In vivo* imaging of malaria parasites — recent advances and future directions. *Curr. Opin. Microbiol.* **8**, 407–414 (2005).
2. Heussler, V. & Doerig, C. *In vivo* imaging enters parasitology. *Trends Parasitol.* **22**, 192–195 (2006).
3. Franke-Fayard, B. *et al*. Murine malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unlinked to sequestration. *Proc. Natl. Acad. Sci. USA* **102**, 11468–11473 (2005).
4. Ho, M. & White, N.J. Molecular mechanisms of cytoadherence in malaria. *Am. J. Physiol.* **276**, C1231–C1242 (1999).
5. Sherman, I.W., Eda, S. & Winograd, E. Cytoadherence and sequestration in *Plasmodium falciparum*: defining the ties that bind. *Microbes Infect.* **5**, 897–909 (2003).
6. Mackintosh, C.L., Beeson, J.G. & Marsh, K. Clinical features and pathogenesis of severe malaria. *Trends Parasitol.* **20**, 597–603 (2004).
7. Hunt, N.H. & Grau, G.E. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol.* **24**, 491–499 (2003).
8. Janse, C.J. & Waters, A.P. *Plasmodium berghei*: the application of cultivation and purification techniques to molecular studies of malaria parasites. *Parasitol. Today* **11**, 138–143 (1995).
9. Janse, C., Ramesar, J. & Waters, A.P. High efficiency transfection and drug-selection of genetically transformed blood stages of the rodent malaria parasite, *P. berghei*. *Nat. Protocols* **1**, 346–356 (2006).
10. Kirchgatter, K. & Del Portillo, H.A. Clinical and molecular aspects of severe malaria. *An. Acad. Bras. Cienc.* **77**, 455–475 (2005).
11. Cowman, A.F. & Crabb, B.S. Invasion of red blood cells by malaria parasites. *Cell* **124**, 755–766 (2006).
12. Gilks, C.F., Jarra, W., Harvey-Wood, K., McLean, S.A. & Schetters, T. Host

diet in experimental rodent malaria: a variable which can compromise experimental design and interpretation. *Parasitology* **98**, 175–177 (1989).
13. Ho, M., Hickey, M.J., Murray, A.G., Andonegui, G. & Kubes, P. Visualization of *Plasmodium falciparum*–endothelium interactions in human microvasculature: mimicry of leukocyte recruitment. *J. Exp. Med.* **192**, 1205–1211 (2000).
14. Sadikot, R.T. & Blackwell, T.S. Bioluminescence imaging. *Proc. Am. Thorac. Soc.* **2**, 537–532 (2005).
15. Welsh, D.K. & Kay, S.A. Bioluminescence imaging in living organisms. *Curr. Opin. Biotechnol.* **16**, 73–78 (2005).
16. Ntziachristos, V., Ripoll, J., Wang, L.V. & Weissleder, R. Looking and listening to light: the evolution of whole-body photonic imaging. *Nat. Biotechnol.* **23**, 313–320 (2005).
17. Dufresne, A.T. & Gromeier, M. A nonpolio enterovirus with respiratory tropism causes poliomyelitis in intercellular adhesion molecule 1 transgenic mice. *Proc. Natl. Acad. Sci. USA* **101**, 13636–13641 (2004).
18. Helmchen, F. & Denk, W. Deep tissue two-photon microscopy. *Nat. Methods* **2**, 932–940 (2005).
19. Dunn, K.W. & Young, P.A. Principles of multiphoton microscopy. *Nephron Exp. Nephrol.* **103**, e33–e40 (2006).
20. de Souza, J.B. & Riley, E.M. Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. *Microbes Infect.* **4**, 291–300 (2002).