Chapter 25

Bioluminescence Imaging of *P. berghei* Schizont Sequestration in Rodents

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Abstract

We describe a technology for imaging the sequestration of infected red blood cells (iRBC) of the rodent malaria parasite *Plasmodium berghei* both in the bodies of live mice and in dissected organs, using a transgenic parasite that expresses luciferase. Real-time imaging of sequestered iRBC is performed by measuring bioluminescence produced by the enzymatic reaction in parasites between the luciferase enzyme and its substrate luciferin injected into the mice several minutes prior to imaging. The bioluminescence signal is detected by a sensitive I-CCD photon-counting video camera. Using a reporter parasite that expresses luciferase under the control of a schizont-specific promoter (i.e., the *ama-1* promoter), the schizont stage is made visible when detecting bioluminescence signals. Schizont sequestration is imaged during short-term infections with parasites that are synchronized in development or during ongoing infections. Real-time in vivo imaging of iRBC will provide increased insights into the dynamics of sequestration and its role in pathology, and can be used to evaluate strategies that prevent sequestration.

Key words: Malaria, *Plasmodium berghei*, Schizonts, Sequestration, Adherence, In vivo imaging, Luminescence, Luciferase, Luciferin

1. Introduction

Recent advances in genetic modification of malaria parasites and in vivo imaging technologies to visualize cells expressing reporter genes have significantly broadened the possibilities for in vivo studies examining interactions of malaria parasites with their hosts using small animal models. This protocol describes a methodology for real-time imaging of schizont sequestration of the malaria parasite *Plasmodium berghei* in mice. Infected RBC of different species of

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malaria parasites adhere to endothelial cells of microvasculature of deep tissues (1, 2). Sequestration of iRBC occurs in small blood capillaries of specific organs, such as the brain and lungs, and has been associated with mechanical obstruction of blood flow and vascular endothelial cell activation that may lead to pathology (3–13).

The application of in vivo imaging techniques in laboratory animals has revealed that schizonts of *P. berghei* ANKA also have a distinct sequestration phenotype (14–16), responsible for the disappearance of all schizogonic stages from the peripheral blood circulation. Real-time in vivo imaging showed that the lungs, adipose tissue, and the spleen are the major organs in which *P. berghei* schizont specifically accumulate (14, 17, 18) and evidence has been presented that the host cell receptor CD36, which is expressed on the surface of endothelial cells lining blood vessels of different organs, mediates sequestration. In addition to CD36-dependent schizont sequestration, real-time in vivo imaging of iRBC has provided evidence that iRBC accumulate during severe disease in different tissues, including the brain, resulting in increased parasite loads in these tissues (14, 17, 19–23). It is expected that the application of real-time in vivo imaging of *P. berghei* iRBC will provide increased insights into the dynamics of sequestration and its role in pathology, and can be used to evaluate strategies that prevent sequestration (24, 25).

This protocol describes a technology for imaging of sequestration of *P. berghei* schizonts in organs of live mice, making use of a transgenic parasite line (*Pb*GFP-Luc$_{ama1}$) that expresses the bioluminescent reporter protein luciferase under the control of the schizont-specific *ama-1* promoter. In blood stages, this reporter line expresses luciferase only in the schizonts and very young ring forms and therefore is especially suitable to analyze schizont sequestration, particularly in synchronized infections (14, 18, 26). Other reporter *P. berghei* lines that express luciferase under the constitutive *eef1α* promoter have been used to study parasite distribution in live mice or in extracted organs (19, 21, 23, 27–29). In such lines, all blood stages including gametocytes are luminescent and therefore it is not possible to discriminate between sequestered schizonts and other blood-stage parasites such as rings and trophozoites. Such reporter parasites are therefore less suitable for analysis of schizonts sequestration but are useful tools for quantification of total parasite loads in different organs (see Table 1 for different reporter parasite lines that have been used for imaging of iRBC in mice). The protocol describes: (1) imaging of sequestered schizonts in whole bodies of mice with short-term synchronized infections, (2) imaging of sequestered schizonts in mice with ongoing infections, and (3) imaging of sequestered schizonts in isolated, non-fixed organs. These methods are based on previously described technologies (14, 18). Here, we report an updated protocol based

Table 1
Different *P. berghei* luciferase expressing reporter parasites that are used for real-time imaging of schizont sequestration and parasite distribution in live mice (adapted from ref. (26))

Reporter parasite RMgmDB no.[a]	Luciferase expression controlled by	Notes
P. berghei ANKA *RMgm-30* and *32*[a]	Schizont-specific *ama1* promoter	Analysis of sequestration of schizonts in synchronized infections. Bioluminescence of sequestered schizonts (also newly invaded ring forms show luminescence resulting from carryover of luciferase from mature schizonts)
P. berghei ANKA *RMgm-28* and *29*[a]	Constitutive "all stages" *eef1a* promoter	Analysis of tissue distribution of iRBC. All stages are bioluminescent. These lines produce gametocytes that can complicate tissue distribution analyses as a result of high luminescence signals derived from circulating female gametocytes
P. berghei ANKA *RMgm-333*[a]	Constitutive "all stages" *eef1a* promoter	Analysis of tissue distribution of asexual blood stages. All stages are bioluminescent. This line does *not* produce gametocytes
P. berghei K173 *RMgm-375*[a]	Schizont-specific *ama1* promoter	Bioluminescence of schizonts (also newly invaded ring forms show luminescence resulting from carryover of luciferase from mature schizonts. Schizonts of this line do not sequester and this line does *not* produce gametocytes)
P. berghei K173 *RMgm-380*[a]	Constitutive "all stages" *eef1a* promoter	All stages are bioluminescent. Schizonts of this line do not sequester and this line does *not* produce gametocytes
P. berghei NK65 line 1555cl1[b]	Constitutive "all stages" *eef1a* promoter	Analysis of tissue distribution of iRBC. All stages are bioluminescent. These lines produce gametocytes that can complicate tissue distribution analyses as a result of high luminescence signals derived from circulating female gametocytes
P. berghei NK65 line 1556cl1[b]	Schizont-specific *ama1* promoter	Analysis of sequestration of schizonts in synchronized infections. Bioluminescence of sequestered schizonts (also newly invaded ring forms show luminescence resulting from carryover of luciferase from mature schizonts)

[a]These reporter parasites have been published in the RMgm-database (www.pberghei.eu) of genetically modified rodent malaria parasite mutants
[b]These reporter lines (generated using the "New York" NK65 parasites) are available from the Leiden Malaria Research Group http://www.lumc.nl/con/1040/81028091348221/810281121192556/

on additional studies (17) and unpublished observations as well as experiences from other researchers that have used luminescent parasites to quantify parasite distribution during the course of infection (21, 27–29).

2. Materials

2.1. Parasites

1. *P. berghei*, clone 15cy1 of the ANKA strain. This parasite is used as a reference "wild-type" line of the ANKA strain of *P. berghei*.
2. The reporter parasite line *Pb*GFP-Luc$_{ama1}$ (1037m1f1m1cl1; see Note 1) is used for the in vivo imaging experiments. It expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the control of the schizont-specific *ama-1* promoter (17). For details of *Pb*GFP-Luc$_{ama1}$, we refer to the RMgm-32 database (http://www.pberghei.eu/index.php?rmgm=32).

2.2. Laboratory Animals

In our laboratory, mice of the following two strains are used: Swiss (OF1 ico, Construct 242, aged 6 weeks, 25–26 g) and C57BL/6 (C57Bl/6Jico OF1, Construct 1, aged 6 weeks, 20–25 g). The mice are obtained from Charles River (see Note 2). Other (including inbred and transgenic) mice can also be used for in vivo imaging experiments.

2.3. Reagents

1. Purified *P. berghei* schizonts of parasite line *Pb*GFP-Luc$_{ama1}$. The protocol requires the generation of $0.5–1 \times 10^8$ cultured and purified schizonts to establish a synchronous infection in four mice. Reagents and equipment required for preparation of the purified, mature, and viable schizonts are not described in this chapter. For these details, we refer to the protocols describing culture and purification of *P. berghei* schizonts (18, 30).
2. Methanol.
3. Giemsa solution (Merck, cat. no. 1666 789); working solution, 10% Giemsa solution in Sörensen staining buffer.
4. Phosphate-buffered saline (PBS). PBS stock solution 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl; pH 7.0. For a working solution, dilute the stock solution with 9 volumes of distilled water. Adjust the pH to 7.2 with 1 M HCl and sterilize by autoclaving for 20 min at 120°C.
5. D-Luciferin sodium salt. Dissolve 1 g D-luciferin in 12.5 ml PBS to give a stock solution of 80 mg/ml 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 body weight (i.e., 30 µl for a mouse of 20 g).
6. Heparin solution (Sigma, cat. no. H3149). Prepare the heparin stock solution at 300 U/ml in distilled H_2O and store at –20°C in 1-ml aliquots. The working solution can be stored at 4°C for a few weeks.

2.4. Equipment

1. Biohazard Class II safety cabinet (see Note 3).
2. IR heat lamp (homemade or from Science Products).
3. Anesthesia system: used for anesthesia of mice prior to and during the intravenous (iv) injection of purified schizonts.
4. Insulin syringes: MicroFine+, 0.5 ml; 0.30 mm (30 G)×8 mm (Becton Dickinson, cat. no. 324870).
5. Butterfly needles (winged needles; 21 g, green label or 23 g, blue label) used for perfusion experiments.
6. Peristaltic pump (Bio-Rad, cat. no. Model EP-1 Econo Pump 731-8140EDU) and 3.2-mm PharMed tubing for the pump (Bio-Rad, cat. no. 731-9007EDU).
7. A mouse holder for the perfusion experiments.
8. Scissors, scalpel, forceps (used for collection of organs and perfusion experiments).
9. Artagain paper: nonfluorescent black paper placed on the imaging platform of the in vivo imaging system to reduce background noise.
10. Black nonfluorescent tape (to reduce background noise) for fixing the mice during imaging and imaging of isolated organs.
11. Black plastic container (such as weighing boat) used for imaging of the isolated organs.
12. Light microscope: all light microscopes with an 100× oil-immersed objective are suitable for this purpose.
13. In vivo imaging system (i.e., Lumina II from Caliper). All in vivo imaging systems with bioluminescent imaging option are suitable.
14. Anesthesia system (i.e., XGI-8 gas connected to the Lumina II from Caliper): used for anesthesia of mice prior to and during in vivo imaging. Mice are anesthetized in the "induction chamber," which is prefilled 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.
15. Contura HS-40 shaver (Wella): used for removal of the fur from the skin of mice by shaving (Optional).

2.5. Software

1. Imaging data are analyzed with the software provided with the in vivo imaging system (i.e., LIVING IMAGE 4.2 for the Lumina II from Caliper).
2. Microsoft Excel is used to conduct preliminary data analyses.
3. GraphPad Prism software (Graph-Pad software, Inc., USA) or comparable software is used for statistical analyses.

3. Methods

3.1. Imaging of Sequestered Schizonts in Whole Bodies of Mice with Short-Term Synchronized Infections of Parasite PbGFP-Lu$_{cama1}$

3.1.1. Infection of Four Mice (Day 0 at Noon)

1. Mix 0.5–1 × 10^8 cultured and purified schizonts in 800 μl of complete culture medium in a Biohazard Class II safety cabinet (with care because schizonts are fragile). The schizonts of parasite line *Pb*GFP-Luc$_{ama1}$ are obtained as described in (18, 30).
2. Place mice under an IR heat lamp (5–10 min before injection of the parasites). The tail veins swell at higher temperature, simplifying the iv injection procedure. In addition, prepare gas anesthesia of the in vivo imaging 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.
3. With a MicroFine + insulin syringe, inject 200 μl of the schizont suspension into a tail vein of each of the four mice under anesthesia. The injection of schizonts should be performed at 12:00 (see Note 4). This time point corresponds to $t = 0$ of the synchronous infection.
4. Make a thin blood smear on a microscope slide from one droplet of tail blood from each of the four infected mice at 4:00 pm ($t = 4$). Fix the smears for 2 s with methanol and stain with a fresh Giemsa working solution for 10 min. Wash the slide with tap water. 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.
5. If the parasitemia ranges between 0.5 and 3%, proceed with Subheading 3.1.2, steps 1–10 (if not, see Note 5).

3.1.2. Imaging Sequestered Schizonts

Imaging can be performed on whole bodies of mice at various time points or on dissected organs of mice (see Subheading 3.3). The latter option should be used if a more detailed image of sequestration in individual organs is required (see Fig. 1 for the workflow).

1. Imaging sequestered schizonts at day 0 (11:00 pm). The first time point at which the bioluminescence signal of the infected mice is measured is 11:00 pm. 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 (optional). Removal of the fur is performed to prevent quenching

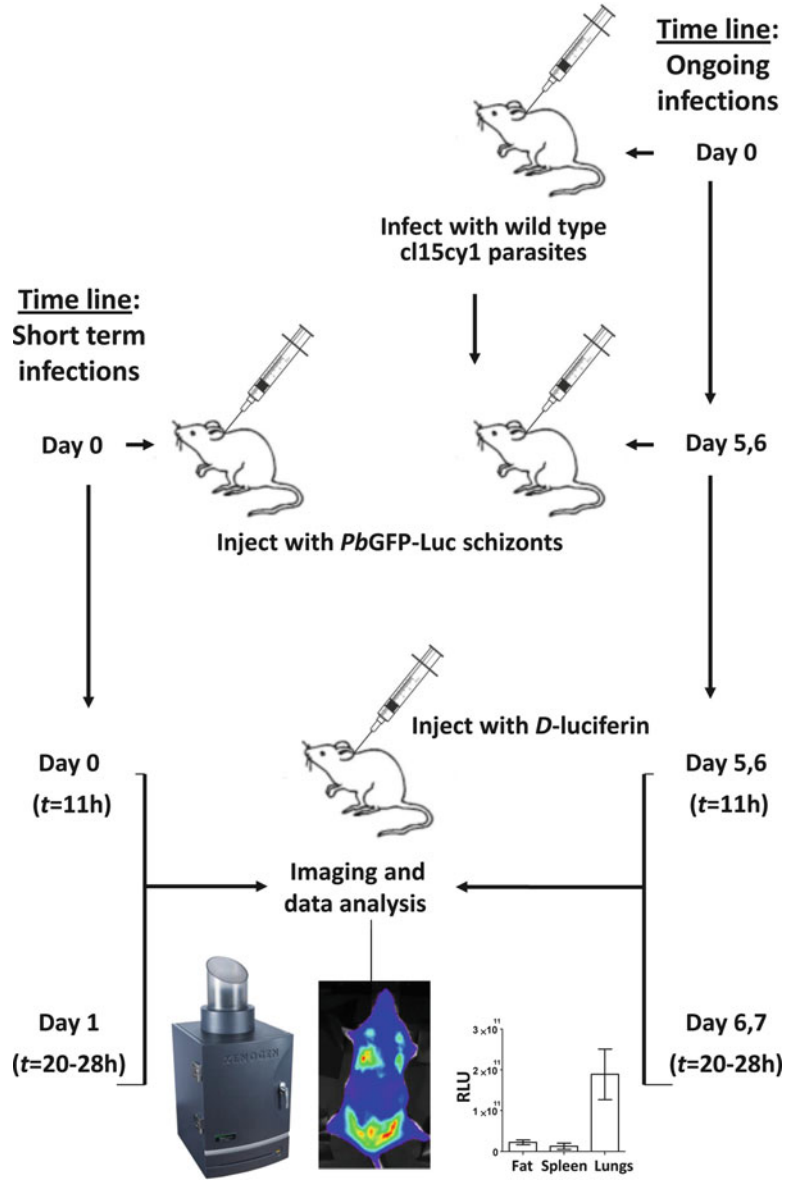

Fig. 1. Workflow scheme for imaging schizont sequestration in mice with short-term and with ongoing infections. *RLU* relative luminescence units.

of the light signal, and must be done carefully as a hematoma might influence imaging.

2. Prepare the in vivo imaging system for imaging mice (or organs) at $t=11$ (Fig. 2 shows the Lumina II and anesthesia system from Caliper) (see Note 6).
3. Inject 30 µl D-luciferin substrate solution subcutaneously into scruff of the neck of the anesthetized mouse (see Note 7).

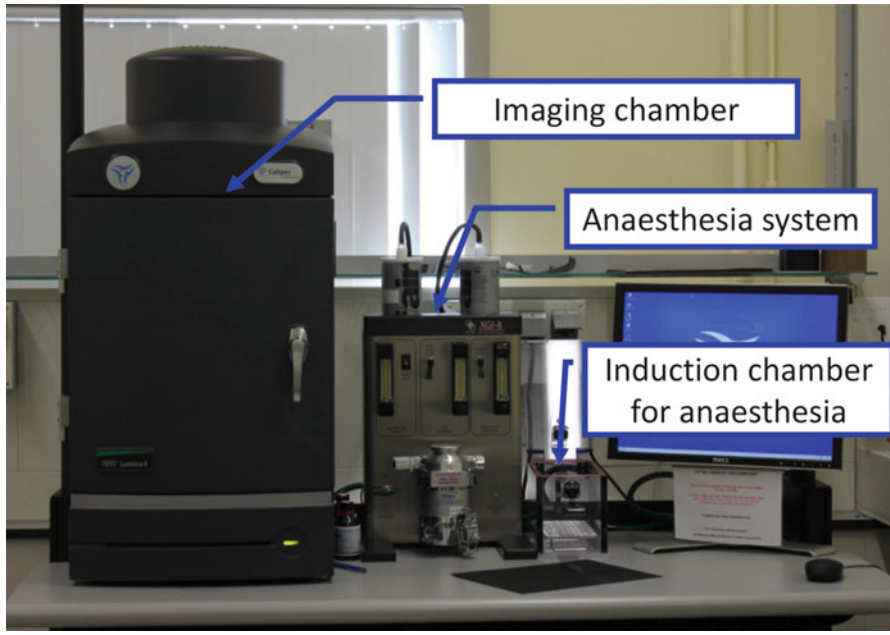

Fig. 2. As an example, the in vivo imaging system "IVIS Lumina II" (Caliper) is shown that has been used in our laboratory for real-time imaging of *P. berghei* iRBC in whole bodies of live mice and in isolated, non-fixed organs.

4. Place the mouse on a piece of Artagain paper and position it under the camera in the center of the sample stage (if needed, fix with black tape). The gated sample stage is pre-warmed to 37°C and thereby stabilizes the body temperature of the mouse (see Note 8).
5. Wait for 3 min before acquiring the bioluminescence image. This period allows circulation of the D-luciferin substrate within the whole body of the mouse.
6. Acquire the bioluminescent image. The bioluminescent signal collected is linearly related to the exposure time within a range of 5 s to 10 min. Routinely, mice are imaged for 10–60 s when mice have been injected with 200 µl of the suspension with $0.5–1 \times 10^8$ schizonts (see Subheading 3.1.1, steps 1–3). If necessary, acquire a new image with shorter or longer exposure time.
7. After exposure is complete, the overlay of the photographic and luminescent picture is displayed (see Fig. 3 for a representative image of sequestered schizonts in a whole mouse).
8. Save imaging data for post-processing analysis (i.e., measurement of the intensity of bioluminescent signals in a specific area (see Subheading 3.1.3)).
9. Remove the mouse from the imaging chamber and repeat steps 3–8 for a new mouse. When all images have been recorded, proceed to step 10.

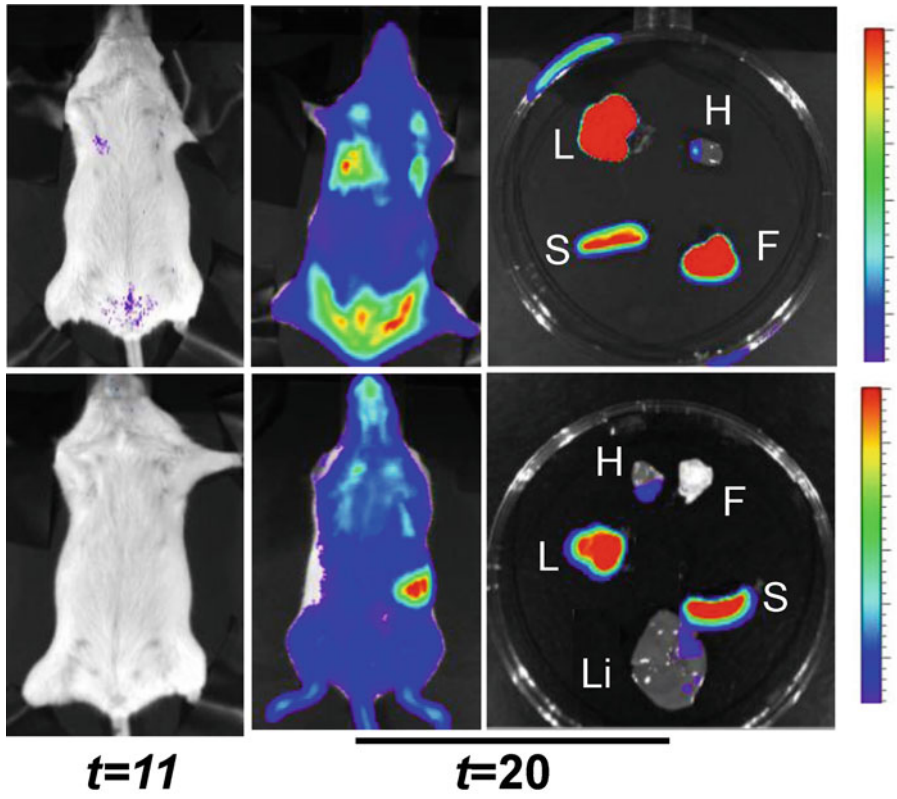

Fig. 3. Representative images of schizont sequestration in whole bodies of mice and in isolated, non-fixed organs. Images are acquired at different time points after injection of purified mature schizonts. At $t=11$, trophozoites are present in the blood that do no sequester and do not express luciferase, resulting in the absence of luminescence signals. In wild-type *P. berghei* ANKA parasites, luciferase-expressing schizonts accumulate in the lungs, adipose tissue and the spleen at $t=20$ (*upper panel*). In the *lower panel*, a mouse is shown that is infected with parasites of a laboratory line, *P. berghei* K173 strain, which does not sequester. At $t=20$, schizonts are in the peripheral blood circulation and accumulate in the spleen (adapted from (26)). *F* fat, *H* heart, *L* lungs, *Li* liver, *S* spleen.

10. Imaging of sequestered schizonts (day 1). Repeat steps 3–9 at 8:00 am ($t=20$), 10:00 am ($t=22$), and 4:00 pm ($t=28$) to record the bioluminescence images for all mice. The optimal time period to image schizonts sequestered in the blood capillaries of the inner organs is between 20 and 22 h ($t=20$–22) after injection of the schizonts (see Note 9). At $t=28$, most schizonts have ruptured and merozoites have invaded new erythrocytes. These newly invaded erythrocytes, containing ring forms do not sequester and are present in the whole blood circulation of the body. Since young ring forms still express GFP-Luciferase, a luminescent signal is detected in the whole body.

3.1.3. Image Analysis

The whole-body bioluminescence images of mice provide 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 are used during the experiments. Most in vivo imaging system softwares contain tool options that enable the quantification of bioluminescent signal emanating from specific areas of the mouse ("region of interest," ROI).

1. Select the image to analyze.
2. Create ROI on the image. Multiple ROI of different sizes can be created on the same image (i.e., whole body of a mouse, specific area of a mouse or of extracted organs).
3. Determine the bioluminescent intensity in ROI. The measurement of the signals results in the generation of a "measurement table" that contains data on the ROI measurement (total and average photon counts) and ROI information (dimension, size, etc.).
4. Export the data and proceed with statistical analysis (see Note 10).

3.2. Imaging Sequestered PbGFP-Lucama1 Schizonts in Mice with Ongoing Infections with Wild-Type Parasites

The followings protocol describes the analysis of sequestration of *Pb*GFP-Luc$_{ama1}$ schizonts in mice during an ongoing infection with wild-type *P. berghei* ANKA parasites. It is well known that during the course of an infection both the expression of host cell receptors and of parasite ligands involved in sequestration can change, which influences the pattern of sequestration (31, 32). To analyze the dynamics of schizont sequestration during an ongoing infection, mice are first infected with parasites of the wild-type *P. berghei* ANKA reference line cl15cy1. After infection has been established, mice are injected with purified *Pb*GFP-Luc$_{ama1}$ schizonts as described in Subheading 3.1.1, steps 1–3. Injection of these schizonts is performed at day 5 or 6 after the beginning of infection.

3.2.1. Establishing an (Ongoing) Infection with Wild-Type Parasites

1. Collect one to three droplets (4–12 μl) of tail blood in 8 ml PBS from a mouse infected with wild-type *P. berghei* parasites (ANKA strain, clone 15cy1). The parasitemia (i.e., the percentage of iRBC) in this mouse must be in the range of 5–15%.
2. Immediately inject intraperitoneally (ip) 5×10^4 infected erythrocytes (~0.1 ml of the cell suspension per mouse) into four naive mice and proceed with step 1 of Subheading 3.2.2.

3.2.2. Imaging Sequestered PbGFP-Lucama1 Schizonts

1. At day 5 or 6, inject the four mice with purified *Pb*GFP-Luc$_{ama1}$ schizonts as described in Subheading 3.1.1, steps 1–3.
2. Image schizont sequestration at day 6/7 or 7/8, as described in Subheading 3.2.1, steps 1–10. Note that the infected mice will develop an infection with wild-type parasites in which parasitemia increases from 0.01 to 0.1% at day 4 after infection to 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 *Pb*GFP-Luc$_{ama1}$ schizonts at day 5 or 6 of infections. Imaging at day 6 or 7 (and possibly day 8) therefore demonstrates sequestration patterns at a time point just before manifestation of the clinical symptoms.

3. Analyze images as described in Subheading 3.1.3.

3.3. Imaging Sequestered PbGFP-Lucama1 Schizonts in Isolated, Non-fixed Organs

Imaging of isolated organs provides additional data on the precise localization of the signals, and can result in a higher imaging sensitivity by reducing the "quenching effect" from other tissues. Perfusion to remove circulating blood can be performed prior to removing organs (see Subheading 3.3.2, steps 1–5).

3.3.1. Organs Collected Without Perfusion

1. Collect organs from (1) mice with short-term infections of parasite line *Pb*GFP-Luc$_{ama1}$ (see Subheading 3.1, steps 1–14) or (2) from mice with ongoing infections with wild-type parasites and infected with *Pb*GFP-Luc$_{ama1}$ schizonts (see Subheading 3.2).
2. Dissect the desired organs from the anesthetized mouse as quickly as possible and place the organs on black tape or plastic holder (see Note 11).
3. Remove blood traces carefully and wet the organs with PBS to prevent dehydration if you expect to reimage the organs.
4. Acquire the bioluminescent image. The bioluminescent signal collected is linearly related to the exposure time within a range of 5 s to 10 min. Routinely, organs are imaged for 10–60 s when collected from mice that have been injected with 200 µl of the suspension with $0.5–1 \times 10^8$ schizonts (see Subheading 3.1.1, step 1–3). If necessary, acquire a new image with shorter or longer exposure time.
5. After exposure is complete, the overlay of the photographic and luminescent picture is displayed (see Fig. 3 for a representative image of sequestration of schizonts in extracted organs). Save imaging data for post-processing analysis (i.e., measurement of the intensity of the bioluminescent signal in specific areas) (see Subheading 3.1.3 and Note 12).

3.3.2. Organs Collected After Perfusion

Intracardiac perfusion can be performed to remove circulating blood; therefore, the measurements are derived exclusively from schizonts sequestered in tissue microvasculature of organs.

1. Inject 30 µl D-luciferin substrate solution subcutaneously into the scruff of the neck of an anesthetized mouse.
2. Fix the mouse on a polystyrene support to minimize the movement of the animal during the perfusion procedure.

3. Open the thoracic cavity, expose the heart and visualize the ascending aorta. Perform a small incision in the right heart ventricle.
4. Insert a butterfly needle through the left ventricle into ascending aorta.
5. Into the plastic tubing, close to the connection with the butterfly needle, inject 100 μl of heparin stock solution, then start to slowly perfuse (pump speed at 13 ml/min) the mouse with 26 ml of a saline solution (e.g., PBS).
6. Perfusion is complete when the blood-enriched saline solution begins to run clear from the right ventricle, indicating that the circulating blood has been removed. Usually 2 min of perfusion is enough to remove the blood from circulation but if necessary the time of perfusion could be extended for 1 or 2 min. High pump speed or longer perfusion times can lead to organ damage (e.g., the lungs).
7. Collect and image organs as described in Subheading 3.3.1, steps 2–5.

4. Notes

1. The *Pb*GFP-Luc$_{ama1}$ line (1037m1f1m1cl1) stably expresses a fusion protein of GFP and luciferase (GFP-luc) and does not contain a drug-selectable marker in its genome. The *gfp-luciferase* gene is integrated into the silent *230p* genomic locus by double crossover integration. In previous papers, the use of reporter line 354cl4 (14) has been described for analysis of schizont sequestration. We now routinely use line *Pb*GFP-Luc$_{ama1}$ because of the absence of a drug-selectable marker and the stable integration of the reporter gene into the *230p* locus. Line 354cl54 is available from MR4 (http://www.mr4.org) and line 1037m1f1m1cl1 from the Leiden Malaria Research Group (http://www.lumc.nl/con/1040/81028091348221/810281121192556). Table 1 shows different luciferase-expressing *P. berghei* reporter parasites lines that have been used for real-time imaging of schizont sequestration and parasite distribution in live mice.
2. All experiments using mice must be performed according to the applicable national guidelines and regulations. Diets of laboratory rodents with low content of total protein, energy, and/or *p*-aminobenzoic acid (PABA) can negatively influence *P. berghei* infections (33). In our laboratory, we therefore provide diets with high protein content (20–25% of total and gross energy content; 18,000–20,000 kJ/kg). If transgenic mice are

used, care must be taken to ensure that these mice do not also express luciferase and also use D-luciferin as a substrate, as this may mask the signal derived from parasites. Mice are kept under normal (day/night) light conditions.

3. Most manipulations with blood infected with genetically modified *P. berghei* parasites are performed in a Class II safety cabinet.
4. The preparation of purified schizonts determines the timing of injection as schizonts should be injected directly after purification. This time can vary but it is important to note the time of tail vein injection with the purified schizonts and to follow the time course of parasite development and sequestration.
5. Injection of schizonts results in rapid invasion of new erythrocytes within 2–4 h after injection. Usually, parasitemia increases during this period to levels between 0.5 and 3%. However, 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 percentage of reticulocytes present in the blood of the mouse. It is recommended to aim for a parasitemia of 0.5–3%. Lower parasitemia might 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 multiply infected erythrocytes in which parasite development is often impaired.
6. Use the in vivo imaging system as recommended by the manufacturer. Ensure that the system is operational and that the automatic background measurements have been performed with settings that will be used for imaging the mice. Systems are run by specific softwares that also serve as guides to help (inexperienced) users through the steps associated with quantitative in vivo imaging and data analysis.
7. Reproducible imaging results are obtained with subcutaneous injection of the substrate. However, we have evidence that iv injection of D-luciferin might improve imaging sensitivity, especially in organs that can degrade and/or eliminate the substrate more rapidly or are less accessible for the substrate (e.g., the liver).
8. Ensure that the observation field 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 are used for imaging a whole body or isolated organs. Remove all dust particles from the sample stage. Mice can be fixed by taping the legs to prevent

them from moving and interfering with the imaging of organs. Black tape should be used for fixing the mice to prevent background light emission.

9. The optimal time period to image schizonts sequestered in the blood capillaries of the organs is between 20 and 22 h ($t = 20–22$) after injection of schizonts ($t = 0$). After invasion of erythrocytes by merozoites between 0 and 4 h, parasites 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 results in the production of 12–16 merozoites. During the S/M phase (between 18 and 24 h after merozoite invasion), the infected erythrocytes disappear from the blood circulation as a result of adherence of schizonts to endothelial cells of blood capillaries in inner organs, such as lungs, spleen, and adipose tissue (see Fig. 3). When the mature schizonts rupture, merozoites invade new erythrocytes and start the next developmental cycle. Therefore, the optimal time for imaging sequestered schizonts is between 20 and 22 h after blood tail injection of parasites. Since young ring forms (present until 4 h after invasion) still express the GFP-Luciferase, it is important that the sequestration is imaged before the start of the new cycle of reinvasion and development to avoid "background" signals from ring forms present in the peripheral blood circulation. The whole-body bioluminescence images of mice 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 used. At $t = 28$, most schizonts have ruptured and merozoites have invaded new erythrocytes. Erythrocytes containing ring forms do not sequester and are present in the whole blood circulation of the body. Since young ring forms still express GFP-Luciferase, a luminescent signal is detected in the whole body.
10. Bioluminescence imaging is simple to execute, allows monitoring of the course of biological processes without killing the 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 (34–36). Usually, we use the mean luminescent values of bodies/organs of four mice per imaging time point.
11. Just before dissecting organs, inject 30 μl of D-luciferin substrate solution subcutaneously in the scruff of the neck of the anesthetized mouse. If the mouse was previously examined by

whole-body imaging, it is advisable to re-inject D-luciferin prior to imaging of extracted organs. We have evidence that iv injection of D-luciferin might improve imaging sensitivity, especially for organs that can degrade and/or eliminate the substrate more rapidly or are less accessible for the substrate (e.g., the liver).

12. To facilitate the analysis of signal intensities in organs from the different mice, it is recommended to place the organs of the different mice in the same relative order on the plastic black holder and in the same position in the imaging chamber. If bioluminescence signals of the various organs differ significantly in intensity, it is advised to image organs separately (light quenching is higher in "dark" and large organs). This enables to image low signals 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 mice with comparable parasitemia.

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