Chapter 30

Quantitative Analysis of *Plasmodium berghei* Liver Stages by Bioluminescence Imaging

Takeshi Annoura, Séverine Chevalley, Chris J. Janse, Blandine Franke-Fayard, and Shahid M. Khan

Abstract

We describe simple and sensitive in vitro and in vivo assays to analyze *Plasmodium* liver stage development using transgenic *P. berghei* parasites (*Pb*GFP-Luc$_{con}$), which express the bioluminescent reporter protein, luciferase. In these assays, parasite development in hepatocytes is visualized and quantified by real-time bioluminescence imaging both in culture and in live mice. We also describe quantification of in vitro liver-stage development by measuring luminescence using a microplate reader. Reporter-parasite based quantification of liver-stage development is faster and correlates very well with established quantitative RT-PCR methods currently used to assess parasite development inside hepatocytes, both in live mice and in culture.

Key words: Malaria, *Plasmodium berghei*, Sporozoites, Preerythrocytic stages, Liver, Hepatocytes, Luciferase, Luminescence, In vivo imaging, Drug screening, Mice

1. Introduction

Quantitative analysis of *Plasmodium* liver-stage development both in vivo in laboratory rodents and in vitro in cultured liver cells is hampered by the low levels of parasite infection and by the complicated methods required to monitor parasite growth. Currently, one of the standard ways to assess drug efficacy against liver stages is to monitor liver stage development, both in vitro and in vivo, by quantitative RT-PCR (qRT-PCR) methods (1–5), and this is both time-consuming and expensive. Other studies have involved assessing parasite viability and direct quantification of development by microscopy (6), RNA hybridization (7), or infrared fluorescence

Robert Ménard (ed.), *Malaria: Methods and Protocols*, Methods in Molecular Biology, vol. 923,
DOI 10.1007/978-1-62703-026-7_30,

scanning system (8). However, these methods not only are prone to large variations between observers but are also time-consuming given the very low infection rates (generally less than 2%) observed in cultured hepatocytes (8). Here we describe simple and sensitive in vitro and in vivo assays to visualize and quantify liver-stage development using the transgenic *P. berghei* parasites *Pb*GFP-Luc$_{con}$, which expresses the bioluminescent reporter protein, luciferase. The luminescence-based quantification of parasite development in hepatocytes has been shown to correlate very well with established quantitative RT-PCR methods (9). Specifically, analysis of liver infections by whole-body real-time imaging correlates well with quantitative RT-PCR analysis of extracted livers. In addition luminescence-based quantification of liver stage parasites in cultured hepatocytes by real-time imaging or using a microplate reader also correlates well with quantitative RT-PCR methods. Both the in vitro and in vivo liver imaging assays are amenable to screen inhibitors and vaccines against liver stages (9, 10). Real-time imaging of liver stages in mice has been successfully used to examine host factors regulating liver infections and to monitor liver-stage development of genetically attenuated parasites (11). Importantly, the in vivo imaging assays allow the course of an infection to be monitored, both throughout liver-stage parasite development and in the blood stage of infection without sacrificing the animal, and therefore, can greatly reduce the number of experimental animals required to determine drug sensitivity. The simplicity and speed of quantitative analysis of liver-stage development by real-time imaging compared to the PCR-based methodologies, as well as the possibility to analyze parasite development in live mice without surgery, should greatly enhance and simplify analyzing the effect of drugs and vaccines on the liver stage of *Plasmodium*.

2. Materials

2.1. Reporter Parasite

For the assays the reporter parasite line *Pb*GFP-Luc$_{con}$ (676m1cl1) is used, which expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the control of the constitutive *eef1*α promoter (12). For details of *Pb*GFP-Luc$_{con}$, see RMgm-29 (http://www.p*berghei*.eu/index.php?rmgm=29).

2.2. Laboratory Animals

In our laboratory, mice of the following two strains are routinely 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. Other (strains or transgenic) mice can also be used for in vivo imaging experiments (see Note 1).

2.3. Reagents

1. Sporozoites of parasite line *Pb*GFP-Luc$_{con}$. This protocol requires the collection of (large numbers) of sporozoites. Sporozoites are removed from the salivary glands of infected *Anopheles stephensi* mosquitoes at days 20–28 after feeding on mice infected with *Pb*GFP-Luc$_{con}$ parasites. For procedures of maintenance/rearing of mosquitoes and infection of mosquitoes, we refer to ref. (13).
2. Hepatocytes. The human hepatocyte carcinoma cell line Huh7 (JCRB0403, JCRB Cell Bank, JP) is used for in vitro cultures of the liver stages.
3. Fetal bovine serum, heat-inactivated (FBS; Invitrogen; cat. no. 10108-165). Store at –20°C.
4. Phosphate-buffered saline (PBS). PBS stock solution (10×): 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl, pH 7.4. For a working solution, dilute the stock solution with 9 volumes of distilled water, adjust the pH to 7.2 with 1.0 M HCl and sterilize by autoclaving for 20 min at 120°C.
5. Complete RPMI1640 culture medium. RPMI1640 medium (Invitrogen; cat. no. 31870-025) supplemented with FBS to a final concentration of 10 or 20% (v/v), 1% GlutaMAX (Invitrogen; cat. no. 35050) and 1% penicillin–streptomycin (MP Bio; cat. no. 1670049).
6. Trypsin, 0.05% (1×) with EDTA 4Na (Invitrogen; cat. no. 25300-054).
7. Cell culture lysis reagent (CCLR): Luciferase Assay System kit (Promega, cat. no. E1500). For working solution, dilute the "Cell Culture Lysis 5× Reagent" provided in the kit with Milli-Q water.
8. Luciferase assay substrate solution: Luciferase Assay System Kit (Promega, cat. no. E1500). For working solution, mix 1 vial of luciferase assay substrate and 1 vial of 10 ml luciferase assay buffer together. The mixed solution can be stored at –20°C and can be subsequently freeze/thawed multiple times without a significant loss of activity, however, the solution must be kept in the dark at all times.
9. 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).
10. Inhibitors/antimalarial drugs: dissolve the powders in DMSO, sterile Milli-Q water or culture medium in high concentration as stock solution. Store at 4°C or –20°C. For serial dilutions, dilute the working stock solutions with DMSO and/or culture medium (see Note 2).

2.4. Equipment

1. Stereomicroscope (Leica M80) for mosquito dissection. Most stereomicroscopes are suitable.
2. (Upright) light microscope (Leica DM2500 or Carl Zeiss Standard 25 Zeiss) for counting sporozoites. All light microscopes with 40× objective are suitable.
3. Inverted microscope, Leica DMIL for analysis of Huh7 cell cultures. All inverted microscopes with 20× objective are suitable.
4. Carbon dioxide gas source (for anesthesia of mosquitoes).
5. Precision forceps (Original Swiss Dumont precision forceps, cat. no. K342.1). Most thin precision forceps are suitable.
6. Insulin needle syringe (BD Micro-Fine + U-100 insulin, 0.33 mm; 30 G × 8 mm, BD Medical, France). Most thin small needle syringes are suitable.
7. Incubation (moist) chamber (COSMO BIO CO., LTD. cat. no. KMB-10CG). All moist chambers are suitable.
8. Bürker-Türk counting chamber (Carl Roth GmbH, cat. no. T730.1).
9. Tissue grinder of Polypropylene Pestle for 1.5-ml tubes (Carl Roth GmbH, cat. no. P987.1).
10. 75-cm^2 cell culture flask (Corning cell culture flasks; cat. no. CLS3276).
11. CO_2 incubator (Thermo/Forma Scientific CO_2 Water Jacketed Incubators, Model 3121). All CO_2 incubators for cell culture are suitable.
12. Luminescence microplate reader: Wallac Multilabel Counter 1420 (PerkinElmer, NL). Other microplate readers that can measure bioluminescence are suitable.
13. 24-well (Corning; cat. no. CLS3524).
14. 96-well optical flat-bottomed and black-framed microplates (Nalge Nunc Intl.). All 96-well microplates with black frames and clear flat bottoms are suitable for luminescence measurement (see Note 3).
15. Vortex shaker (Ika Labortechnik).
16. Infra-red heat lamp (home-made or from Science Products).
17. Biohazard Class II safety cabinet (see Note 4).
18. Table-top centrifuge (Beckman Coulter Allegra, GS-6 centrifuge). Most table-top centrifuges with a swing-out rotor are suitable but these need to have a carrier assembly for plates (Beckman Coulter; MicroPlus Carrier Assembly for GH-3.8 Rotors cat. no. BK362394).
19. Contura HS-40 shaver (Wella), used for removal of the fur from the skin of mice by shaving (Optional).

20. Anesthesia system (i.e., XGI-8 gas connected to the Lumina II from Caliper) 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/air) 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.
21. IVIS Lumina II System (Caliper Life Sciences, USA). All in vivo imaging system with bioluminescent imaging option are suitable for this purpose.

2.5. Software

1. Imaging data are analyzed with the software provided with the in vivo imaging system (i.e., LIVING IMAGE 4.1 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) is used for statistical analyses (best-fit) effective concentration (EC50) calculation.

3. Methods

3.1. Analysis and Quantification of In Vitro Liver-Stage Development

This protocol requires the collection of *Pb*GFP-Luc$_{con}$ sporozoites from infected *A. stephensi* mosquitoes. Sporozoites are obtained from dissected salivary glands at days 20–28 after blood feeding. Maintenance/rearing and infection of mosquitoes are performed as described in (13). The total number of mosquitoes required is dependent on the experiment and the number of salivary gland sporozoites per dissected mosquito; in our laboratory we usually obtain sporozoites loads of $0.5–1 \times 10^5$ per mosquito (see Fig. 1 for a schematic representation of the workflow involving collection of sporozoites, in vitro culture and analysis of the liver stages).

3.1.1. Collection of Sporozoites

1. Transfer infected mosquitoes into a tissue net covered 50-ml centrifuge tube.
2. Anesthetize mosquitoes with carbon dioxide (blown directly into the tube). Keep the tube containing the anesthetized mosquitoes on ice until dissection.
3. Place the mosquito in one drop of PBS on a glass slide under a stereomicroscope (magnification 5–20×).
4. Carefully remove the mosquito head from thorax using a forceps and an insulin needle. In general the two glands, each with three lobes, will remain attached to the head (see Fig. 2),

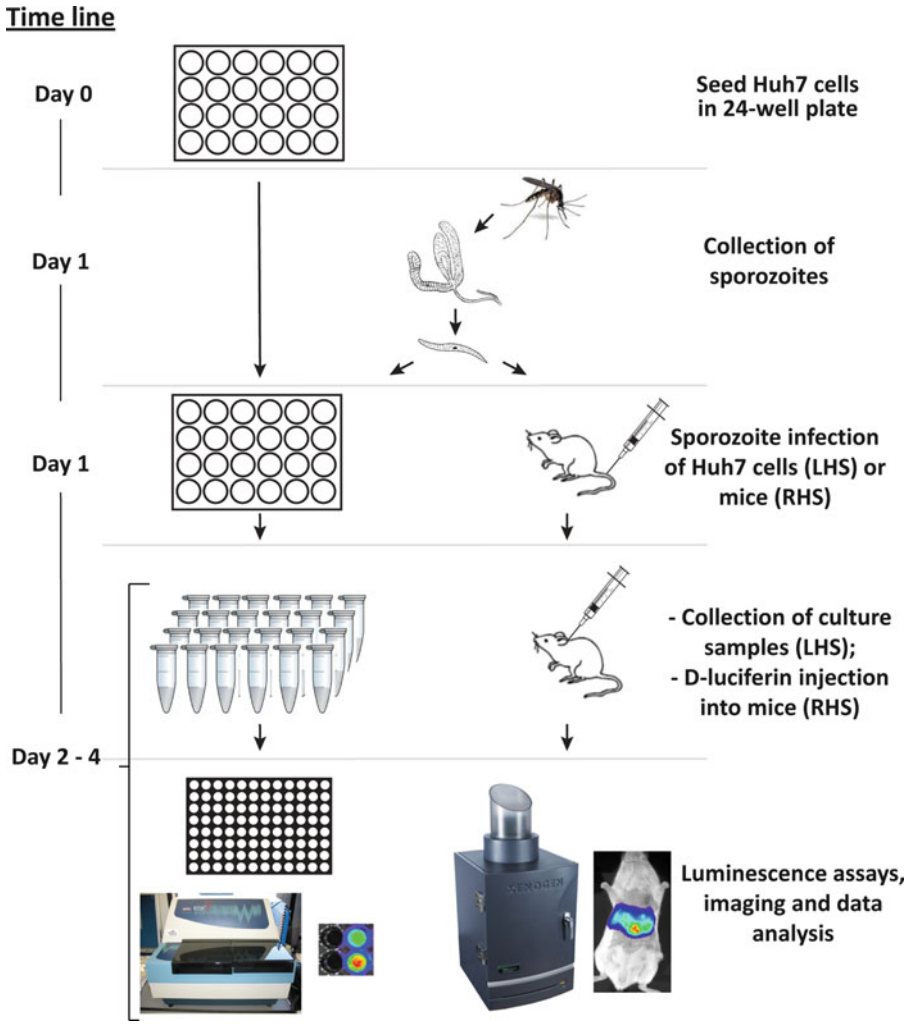

Fig. 1. Workflow scheme for quantitative analysis of *Plasmodium* liver stages. *Left hand side* (LHS): liver-stage development in vitro analyzed by measuring luminescence using a plate reader. *Right hand side* (RHS): liver-stage development in vitro analyzed by real-time bioluminescence imaging of live mice.

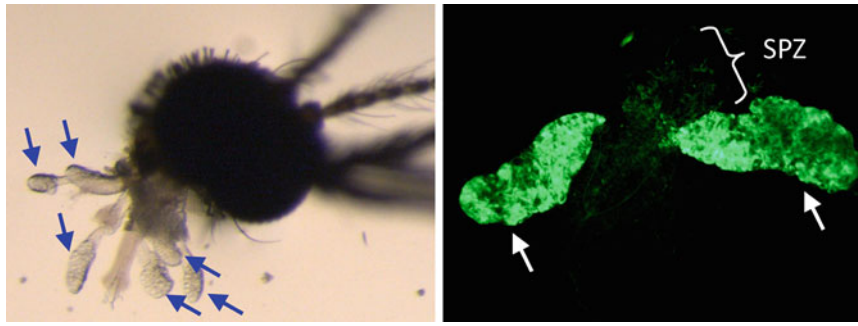

Fig. 2. (**a**) The view of a mosquito's head attached to six intact salivary gland lobes (*grey arrows*; stereomicroscope; .. magnification). (**b**) GFP-expressing sporozoites of parasite line a *Pb*GFP-Luc$_{con}$ in lobes of a salivary gland (*white arrows*) and free sporozoites (SPZ; fluorescence microscope).

but sometimes they remain within the thorax. In this case, gently press the forceps on the thorax in order to release the salivary glands.

5. Collect and transfer the two salivary glands (with six intact lobes) by forceps into a 1.5-ml Eppendorf tube containing 100 μl of incomplete RPMI1640 medium. It is important to pick only the salivary glands without contamination with other mosquito organs.
6. Repeat steps 4–6 until the required number of salivary glands is collected.
7. In order to obtain free sporozoites from the salivary glands, disrupt the collected salivary glands using a PP-pestle homogenizer and Vortex shaker (see Note 5).
8. In order to determine the total number of collected sporozoites, take a 10 μl-sample (from the 100 μl) and place in a Bürker-Türk cell counter.
9. First allow the sporozoites to settle before counting the sporozoites by placing the cell counter for 20 min in a moist chamber.
10. Sporozoite suspensions.
    (a) For the in vitro assays, dilute the sporozoite suspension with complete RPMI1640 medium (10% FBS), to a final concentration of $0.3–10 \times 10^5$ sporozoites per 100 μl for transfer to the Huh7 cultures (see Subheading 3.1.3, step 2).
    (b) For the in vivo assays, dilute the sporozoite suspension with incomplete RPMI1640 medium to a final concentration $1–200 \times 10^3$ sporozoites per 50–800 μl for injection into mice (see Subheading 3.2.2, step 4).

3.1.2. Culture of Huh7 Cells

For principles of Huh7 cell culture, such as storage, thawing and maintenance of cells, see the Japanese Collection of Research Bioresources (JCRB) (http://huh7.com/huh7_cell_culture.html). Most manipulations for the in vitro cultivation of Huh7 cells (in combination with *P. berghei* parasites) are performed in a Class II safety cabinet.

1. Defrost frozen stock-solution of 1 ml Huh7 cells (1×10^7 cells in 1 ml of complete RPMI1640 medium, containing 10% DMSO) in 10 ml Cell complete RPMI1640 culture medium (20% FBS) into a 50-ml centrifuge tube.
2. Centrifuge at $13{,}000 \times g$ for 10 min at RT using a tabletop centrifuge and remove the supernatant.
3. Resuspend the cells by adding 30 ml of complete RPMI1640 culture medium (20% FBS) and seed into 75-cm^2 tissue culture flasks.

4. Incubate the flasks for 12–24 h in a CO_2 incubator (37°C, 5% CO_2).
5. After this culture period, eliminate dead cells by removing culture medium and washing the plates with 20 ml PBS. Add 30 ml complete RPMI1640 medium (20% FBS) and incubate the plates for several days (see Note 6).
6. For collecting Huh7 cells for the "sporozoite assays," remove the culture medium from the plates and "detach" the Huh7 cells by adding 3 ml of the trypsin–EDTA 4Na solution for a period of 5–10 min at 37°C.
7. To the (detached) cell suspension add 17 ml complete RPMI1640 medium (10% FBS) and transfer to 50-ml tubes.
8. Spin at 200 × *g* for 10 min at RT in a table-top centrifuge, remove the supernatant, and resuspend the cells in 10 ml complete RPMI1640 medium (10% FBS).
9. Collect a sample of 10 μl for determination of the numbers of Huh7 cells by counting in a Bürker-Türk counting chamber.
10. Adjust the volume of the Huh7 cell suspension with complete RPMI1640 medium to obtain a final concentration of 5×10^4 cells per ml.
11. Seed 5×10^4 Huh7 cells by adding 1 ml of the final Huh7-cell suspension to wells of a 24-well plate.
12. Before adding sporozoites to the wells, incubate the 24-well plates for 12–24 h in a CO_2 incubator (37°C; 5% CO_2). Huh7 cells will cover 40–60% of the well.

3.1.3. Sporozoite Invasion and Culture of the Liver Stages in Huh7 Cells (with or Without Addition of Inhibitors)

1. Remove the culture medium from the wells of the 24-well plates (see Subheading 3.1.2, steps 12–13) and subsequently add 900 μl fresh, pre-warmed (37°C) complete RPMI1640 medium (10% FBS). See Note 7 for additional information about the timing of adding inhibitors/drugs to the cultures.
2. Add 100 μl of the sporozoite suspension containing $0.3–10 \times 10^5$ sporozoites (see Subheading 3.1.1, step 10) to each well.
3. Spin at 13,000 × *g* for 5 min at RT (table-top centrifuge; Beckman Coulter Allegra, GS-6 centrifuge with carrier assembly for plates swing unit).
4. Incubate at 37°C and 5% CO_2 for 2–3 h.
5. Remove free sporozoites from the wells by removing the medium and replacing it with 1 ml of fresh pre-warmed complete RPMI1640 medium. See Note 7 for additional information about the timing of adding inhibitors/drugs to the cultures.
6. Return plates to the incubator at 37°C and 5% CO_2.

3.1.4. Quantification of In Vitro Liver-Stage Development (with or Without Addition of Inhibitors)

Parasite liver-stage development in Huh7 hepatocytes is analyzed over a 52-h period after the addition of sporozoites. After sporozoite invasion, most *P. berghei* parasites develop in 60–64 h into mature liver schizonts. After 64 h, merozoites are released from the hepatocyte as merosomes, packets of 100–200 merozoites surrounded by host cell membrane. Therefore, liver-stage development can be quantified by measuring bioluminescence of lysed cultured cells up to 52 h after sporozoite invasion, using a microplate reader or by measuring bioluminescence directly in culture plates using the IVIS Lumina II system (see Note 8). Usually, experiments are performed in triplicate (3 culture wells per condition or time-point).

1. Remove the culture medium from the wells of the 24-well plates and add 1 ml PBS for washing.
2. Remove PBS and add 100 µl of 1× cell culture lysis reagent (CCLR).
3. Mix the cells and the CCLR by pipetting until all cells are lysed (when the bottom of the wells become clear and the lysis solution is homogenous).
4. Collect the cell lysis solution and transfer lysed cells from each well to 1.5-ml Eppendorf tubes. These samples can be stored at −80°C until ready to perform the luciferase assay.
5. When all samples have been collected and are ready for the luminescence assay, add 100 µl of "luciferase assay substrate solution" and 10 µl of the lysed cell samples into wells of a black-framed 96-well plate. Samples containing uninfected Huh7 cells are used as negative controls.
6. Measure the light reaction of each well for 10 s using a microplate luminometer. The luciferase activities are expressed as relative luminescence units (RLU) for each sample.
7. Export the data and proceed with statistical analysis of the data.

3.2. Analysis and Quantification of In Vivo Liver-Stage Development

This protocol requires *Pb*GFP-Luc$_{con}$ sporozoites collected from salivary glands of infected *A. stephensi* mosquitoes at days 20–28 after blood feeding. Maintenance, rearing and infection of mosquitoes are performed as described in (13). The total number of mosquitoes required is dependent on the experiment and the number of salivary gland sporozoites per dissected mosquito; in our laboratory we usually obtain sporozoites loads of $0.5–1 \times 10^5$ per mosquito (see Fig. 1 for a schematic representation of the work flow involving collection of sporozoites, infection of mice with sporozoites and analysis of liver stage development).

3.2.1. Preparation of Sporozoites

Sporozoites of the *Pb*GFP-Luc$_{con}$ line are obtained from dissected salivary glands from infected mosquitoes as described in Subheading 3.1.1.

3.2.2. Infection of Mice with Sporozoites

1. Place the mice under an IR heat lamp 5–10 min before injection of the sporozoites (see step 3). The tail veins swell at the higher temperature, simplifying the intravenous injection procedure. In addition, prepare anesthesia system in sufficient time, such as filling the "induction chamber" with the anesthetic vapor (isofluorane/air), to be able to inject the sporozoites immediately after the purification procedure.
2. Prepare the sporozoite suspensions as described in Subheading 3.1.1, step 10.
3. Dilute the sporozoite suspension with incomplete RPMI1640 medium, to a final concentration of $1–200 \times 10^3$ sporozoites per 200 μl for injection into mice. This sporozoite suspension is injected intravenously into the tail vein (see Note 9).

3.2.3. Quantification of Liver-Stage Development In Vivo (with or Without Drug Treatment of Mice)

1. Prepare the in vivo imaging system for imaging the mice (Fig. 3a shows the Lumina II and anesthesia system from Caliper) (see Note 10).
2. For imaging liver stages, anesthetize infected mice at different time points after sporozoite inoculation (e.g., 24, 40, 48 or 64 h) using the isofluorane-anesthesia system.
3. 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.
4. Inject 30 μl D-luciferin substrate solution subcutaneously into the neck of the anesthetized mouse (see Note 11).
5. Place the mouse on a piece of Art again paper and position it under the camera in the centre 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 12).
6. Wait for 3 min before acquiring the bioluminescence image. This period allows circulation of the *D*-luciferin substrate within the body of the mouse.
7. 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, we image infected mice for 60–180 s when mice have been injected intravenously with 1×10^4 sporozoites. If necessary, acquire a new image with shorter or longer exposure time.

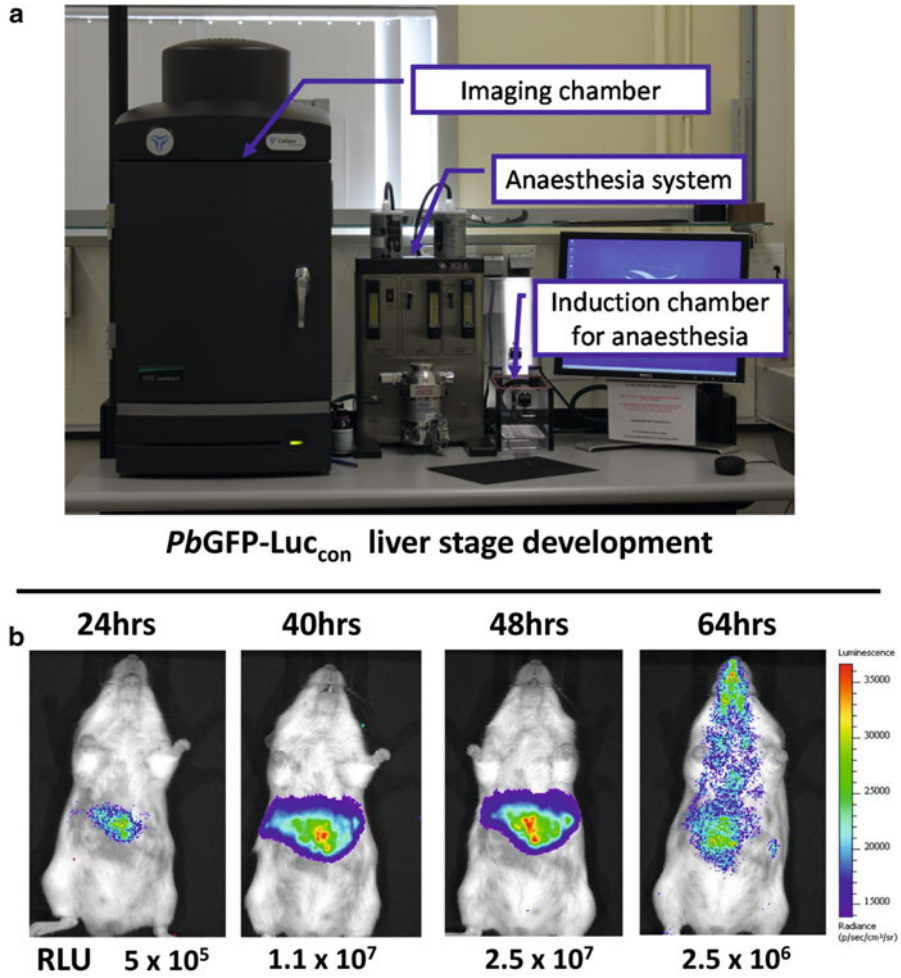

Fig. 3. (**a**) As an example, the in vivo imaging system "IVIS Lumina II" from Caliper is shown, which has been used in our laboratory for real-time imaging of *P. berghei* liver stages in whole bodies of live mice and in isolated, nonfixed livers. (**b**) In vivo images of the same mouse with a developing liver-stage infection, at different time points after intravenous inoculation of 10,000 *Pb*GFP-Luc$_{con}$ sporozoites. At 64 h, merozoites are released from the liver into the blood circulation. The relative luminescence units (RLU), at each time point, are shown under the picture of the mouse.

8. After exposure is complete, the overlay of the photographic and luminescent picture is displayed. See Fig. 3b for representative images of luminescent signals of liver stages in mice infected with *Pb*GFP-Luc$_{con}$ sporozoites at different time points after infection.
9. Save imaging data for post-processing analysis (i.e., measurement of the intensity of bioluminescent signals in a specific area (see Subheading 3.2.4)).
10. Remove the mouse from the imaging chamber and repeat steps 3–8 for a new mouse.

3.2.4. Image Analysis

The whole-body bioluminescence images of mice provide a qualitative assessment of the load/intensity of *Pb*GFP-Luc$_{con}$ liver stages within an animal and this liver load can be directly compared between different animals if the same measurement settings during the experiments are maintained. Most in vivo imaging systems software 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 region of interest (ROI; Liver) on the image (see Fig. 3b).
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 eventually proceed with statistical analysis (see Note 13).

4. Notes

1. 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 (14). 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, they should not express luciferase gene(s) that use D-luciferin as a substrate. Mice are kept under normal (day/night) light conditions.
2. It is very important to dissolve inhibitors/antimalarial drugs completely; vortexing and/or sonication and/or 37°C incubation can help to dissolve compounds. The inhibitors in stock solution can be diluted with DMSO or culture medium according to their properties. It is better to make serial dilutions fresh, though they can also be stored at −20 or −80°C. We prepare stock solutions at a 100 times the final concentration required in the well so that when the drug is added to the well the concentration of DMSO in overnight culture is <1%, which is not harmful to the parasite development. No inhibition controls (i.e., culture without inhibitors) also contain <1% DMSO.

3. For luminescence measurements, we routinely use black-framed microplates as they best reduce light scattering between wells, which can artificially increase the signals detected in neighboring wells and thereby calculated EC50 values.
4. Most manipulations with Huh7 cells and with genetically modified *P. berghei* parasites are performed in a Class II safety cabinet.
5. It is important to maximize the release of sporozoites from the dissected salivary glands. However, one should not homogenize the salivary gland sample too much as this can reduce the number of sporozoites. We therefore do the following: 10 s vortexing, ten strokes of the homogenizer, 10 s vortexing.
6. Usually we check the plates regularly (once a day) under the inverted microscope (10–20× magnification) for the cell growth in these plates. Huh7 cells are usually collected for the sporozoite assays when cells are 70–80% confluent.
7. If you aim to analyze the effect of inhibitors on sporozoite invasion, inhibitors can be added to the 24-well culture plates during this step. If you aim at analyzing the effect specifically on the development of the liver stages (after invasion of hepatocytes by the sporozoites) inhibitors should be added during Subheading 3.1.3, step 5. See also Note 3 for more details on adding inhibitors to the cultures.
8. Liver-stage development can be quantified by measuring bioluminescence in lysed culture samples using a microplate reader (see Subheading 3.1.4, steps 1–7) or by measuring bioluminescence directly in culture plates using the IVIS Lumina II system. For direct imaging we directly add D-Luciferin (80 mg/ml) to the wells wait for 3 min and image the plates in the IVIS Lumina II system in a comparable way as described for imaging infected mice (see Subheading 3.2.3, steps 7–9).
9. Usually mice are infected intravenously with defined numbers (i.e., 10^2 to 10^6) of freshly dissected sporozoites. However, other studies may require sporozoites be administered by other routes of administration. For intradermal inoculation we inject 50–100 μl (with varying numbers of parasites) of the sporozoite suspension into the skin at various site of the body (most often the neck). For subcutaneous inoculation we usually inject 500–800 μl (and varying numbers of parasites) of the sporozoite suspension in the scruff of the neck.
10. 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 software that also serves as a guide to help

(inexperienced) users through the steps associated with quantitative in vivo imaging and data analysis.

11. 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.
12. Ensure that the field of view 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. 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.
13. Bioluminescence imaging is simple to execute, allows monitoring of the course of biological processes without killing the experimental animal, and therefore reducing 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. Usually, we use the mean luminescent values of bodies/organs of three mice per imaging time point.

Acknowledgment

TA, SC, and SMK received financial support of Top Institute Pharma (The Netherlands) project: T4-102.

References

1. Bruna-Romero O et al (2001) Detection of malaria liver-stages in mice infected through the bite of a single *Anopheles* mosquito using a highly sensitive real-time PCR. Int J Parasitol 31:1499–1502
2. Siau A et al (2008) Temperature shift and host cell contact up-regulate sporozoite expression of *Plasmodium falciparum* genes involved in hepatocyte infection. PLoS Pathog 4:e1000121
3. Li J et al (1991) *Plasmodium berghei*: quantitation of *in vitro* effects of antimalarial drugs on exoerythrocytic development by a ribosomal RNA probe. Exp Parasitol 72:450–458
4. Hobbs CV et al (2009) HIV protease inhibitors inhibit the development of preerythrocytic-stage *Plasmodium* parasites. J Infect Dis 199:134–141
5. Cunha-Rodrigues M et al (2008) Genistein-supplemented diet decreases malaria liver infection in mice and constitutes a potential prophylactic strategy. PLoS One 3:e2732
6. Fisk TL et al (1989) *In vitro* activity of antimalarial compounds on the exoerythrocytic stages of *Plasmodium cynomolgi* and *P. knowlesi*. Am J Trop Med Hyg 40:235–239
7. Schofield L et al (1987) Interferon-gamma inhibits the intrahepatocytic development of malaria parasites *in vitro*. J Immunol 139:2020–2025
8. Gego A et al (2006) New approach for high-throughput screening of drug activity on

Plasmodium liver stages. Antimicrob Agents Chemother 50:1586–1589

9. Ploemen IH et al (2009) Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. PLoS One 4:e7881
10. Mwakingwe A et al (2009) Noninvasive real-time monitoring of liver-stage development of bioluminescent *Plasmodium* parasites. J Infect Dis 200:1470–1478
11. Portugal S et al (2011) Host-mediated regulation of superinfection in malaria. Nat Med 17:732–737
12. Franke-Fayard B et al (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol 137:23–33
13. Sinden RE (1997) Infection of mosquitoes with rodent malaria. in The molecular biology of insect disease vectors. eds Crampton J. M., Beard C. B., Louis C. (Chapman & Hall, London, United Kingdom), pp 261–267.
14. Gilks CF et al (1989) Host diet in experimental rodent malaria: a variable which can compromise experimental design and interpretation. Parasitology 98:175–177