Video Article

In vivo Imaging of Transgenic *Leishmania* Parasites in a Live Host

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Abstract

Distinct species of *Leishmania,* a protozoan parasite of the family *Trypanosomatidae*, typically cause different human disease manifestations. The most common forms of disease are visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). Mouse models of leishmaniasis are widely used, but quantification of parasite burdens during murine disease requires mice to be euthanized at various times after infection. Parasite loads are then measured either by microscopy, limiting dilution assay, or qPCR amplification of parasite DNA. The *in vivo* imaging system (IVIS) has an integrated software package that allows the detection of a bioluminescent signal associated with cells in living organisms. Both to minimize animal usage and to follow infection longitudinally in individuals, in vivo models for imaging *Leishmania* spp. causing VL or CL were established. Parasites were engineered to express luciferase, and these were introduced into mice either intradermally or intravenously. Quantitative measurements of the luciferase driving bioluminescence of the transgenic *Leishmania* parasites within the mouse were made using IVIS. Individual mice can be imaged multiple times during longitudinal studies, allowing us to assess the inter-animal variation in the initial experimental parasite inocula, and to assess the multiplication of parasites in mouse tissues. Parasites are detected with high sensitivity in cutaneous locations. Although it is very likely that the signal (photons/second/parasite) is lower in deeper visceral organs than the skin, but quantitative comparisons of signals in superficial versus deep sites have not been done. It is possible that parasite numbers between body sites cannot be directly compared, although parasite loads in the same tissues can be compared between mice. Examples of one visceralizing species (*L. infantum chagasi*) and one species causing cutaneous leishmaniasis (*L. mexicana*) are shown. The IVIS procedure can be used for monitoring and analyzing small animal models of a wide variety of *Leishmania* species causing the different forms of human leishmaniasis.

Protocol

1. Infection of small animals with transgenic *Leishmania*

1. Parasite lines

Transgenic *Leishmania* spp. parasites expressing luciferase are generated using an episomal or an integrating vector as reported.[1 2] Clonal lines are preferred. Two important points are:

(a) Integrated luciferase is preferred over episomal luciferase, since in theory these parasite lines should better retain the transgene in the absence of drug pressure, i.e. after introduction into a mammal. However even integrated transgenes can be lost at low rates,[3] so it is critical to maintain selective drug pressure on the stock culture. In practice, *Leishmania* spp. often retain episomal elements for extended time periods even *in vivo*, although with variation in plasmid copy number per cell.[4] For either type of transgenic parasite, they should always be passed in vitro one time prior to inoculation into animals to avoid potential drug effects.

(b) The *Leishmania* spp. parasites can lose virulence during in vitro culture. In some species (e.g. *L. donovani, L. infantum chagasi*) this loss can occur rapidly over weeks of culture. In other species (e.g., *L. major, L. mexicana*) virulence is retained longer term, for months to years. Nonetheless, clonal transfectants should be screened to identify those retaining full virulence for small animals. Many labs will serially pass the parasites several times (4 or more) through mice or hamsters to augment virulence prior to use in experiments.

2. Preparation of infective metacyclic stage parasites for infection

The infective form of *Leishmania* spp. that is transmitted by the sand fly to the mammalian host is the metacyclic promastigote.[5] Therefore, animal infections with this form of the parasite are preferred as a model of natural infection. Although sand flies are difficult to maintain, parasites grown in vitro will conveniently undergo metacyclogenesis. The percent of metacyclics present in a culture will vary with the number of passes since isolation from the animal, the culture media and the parasite species and strain.

Metacyclics can be purified by positive or negative selection depending on the species and line of *Leishmania*. Changes in the terminal or side chain carbohydrate residues of the abundant surface lipophosphoglycan (LPG) of *L. major* allow it to be purified by loss of agglutination with the lectin PNA (peanut agglutinin).[5,6] Other *Leishmania* species or *L. major* mutants lacking metacyclic LPG can be purified on a Ficoll step gradient.[7] IVIS can be performed using bulk non-purified cultures, but it should be recognized that these contain a mixture of metacyclic and less infectious forms.

Typically, 15-20% of bulk stationary phase *L. i. chagasi* cultures are recovered as metacyclics using parasites that have been isolated within 3 weeks from a hamster or mouse. Parasites are washed by centrifugation, suspended to the desired concentration in buffer (HBSS or PBS with or without Ca^{2+} and Mg^{2+}), and maintained at 4°C for up to 2 hrs prior to inoculation into the mouse.

3. Choice of route for inoculation of *Leishmania* spp. into the host

The clinical disease manifestations of human leishmaniasis vary depending on both the species of parasite and host factors. There are corresponding differences in murine infection, leading investigators to use different models and routes of infection. For example, species causing human CL (e.g. *L. major, L. mexicana, L. amazonensis*) are often inoculated into mice subcutaneously in the footpad or intradermally in the ear.[8,9] Species causing human VL are often introduced intravenously through a tail vein[10-12] or intradermally in the ear.[13] Tail vein infections have been performed either with hamster-derived amastigotes or with promastigotes. We routinely infect mice with the promastigote form of *L. i. chagasi,* either intradermally in the ear or lateral tarsal region[14] or intravenously.

4. Pre-treatment of mice with anesthetic

Wild-type, transgenic or gene knockout mice can be used. Although not formally, quantified, it seems most likely that nude or albino such as BALB/c mice allow for greater light transmission through tissue compared to mice with dark skin and hair pigment such as C57BL/6.

Injectable anesthestic agents such as a mixture of ketamine plus xylazine [80-100 mg/kg + 10 mg/kg, respectively, intraperitoneally (i.p.)] diluted in saline is one preferred method, since the animals will be lightly anesthetized for at least 10 minutes with a single dose. 100-200ul total volume is injected i.p. using a 25-30 gauge needle. The animals are manually restrained firmly by their dorsal skin with their abdomen up and head pointed down. The needle should be positioned with the bevel up and slightly angled. The tip of the needle should just slightly penetrate the lower left quadrant of the abdominal wall.

An inhalant anesthetic such as isoflurane can alternately be used, but animals will only remain under anesthesia for as long as they are inhaling the anesthetic agent.

5. Infection of mice with *Leishmania* spp. parasites

Once the animals are lightly anesthetized, the infection sites are cleaned with 70% ethyl or isopropyl alcohol. The parasite species, dose and infection route is determined by the investigator. The injection volume should be minimized to prevent excessive tissue damage for intradermal (10 µl) or intramuscular (25-50 µl) infection routes. The allowable injection volumes in mice can be larger for intravenous (100-200 µl), subcutaneous (up to 2 ml) or intraperitoneal (up to 2 ml) infection routes.

Infection routes and volumes used in our experiments include intradermal in the ear pinna (10 µl), subcutaneous in the flank (100-200 µl), intraperitoneal (100-200 µl) or intravenous (100-200 µl). Parasite doses have ranged from 10^2 to 10^7 promastigotes.

2. Bioluminescent imaging of *Leishmania* using the IVIS (*in vivo* imaging system)

1. Preparation of D-luciferin for in vivo bioluminescent imaging

D-Luciferin (Caliper LifeSciences) is reconstituted to a concentration of 15 mg/ml in Dulbecco's PBS with or without Mg^{2+} and Ca^{2+}, and syringe filtered (0.2 µm). Aliquots are frozen at -80°C until use. Prior to injection, luciferin is warmed to 37°C in a water bath.

2. Injection of D-luciferin

The injection site is cleaned and luciferin is introduced into conscious animals by an intraperitoneal injection of a 15 mg/ml luciferin solution in DPBS, at a dose of 150 mg/kg. Luciferin can also be injected into anesthetic-treated animals, but the bioluminescence kinetics may vary slightly.

Once injected into animals, luciferin circulates rapidly. It is useful to empirically determine the optimal time to acquire luminescent data after luciferin injection for each experimental model and for different anatomical locations of the mice, because the kinetics of luciferin distribution may vary. A kinetic curve is generated by acquiring a sequence of replicate images.

3. Animals are lightly anesthetized

Inhalant or injectable anesthetics may be used for the imaging procedure. Isoflurane is simpler to administer at this stage, since most IVIS systems have an attached anesthesia chamber and anesthesia nose cone manifold inside the imaging chamber. The anesthesia is split between the anesthesia chamber and the manifold inside the imaging chamber.

The animals are placed in the clear Plexiglass anesthesia chamber and lightly anesthetized with 2.5-3.5% isoflurane. Animals are visually monitored to ensure an effective degree of anesthesia has been induced, and that their breathing is unhindered. Sufficient degree of anesthesia is verified by lack of withdrawal from painful paw pressure. The amount of isoflurane may be reduced to 1.5-2% after animals are lightly anesthetized.

4. Bioluminescent data is collected

Adequately anesthetized animals are transferred from the anesthesia chamber to the imaging chamber and positioned so that the nose cones attached to the manifold will deliver a continuous and regulated flow of isoflurane.

The Living Image software program (Caliper Life Sciences) is opened and the IVIS is initialized. For our experiments, we use the Xenogen IVIS 200 system (Caliper Life Sciences). The CCD camera will be automatically cooled and maintained at the appropriate temperature after initializing the IVIS. The camera system setup and image acquisition parameters are set in the IVIS System Control Panel. The acquisition parameters are largely based on the number of animals and the intensity of the bioluminescence. Important parameters include: imaging mode (luminescent or fluorescent), exposure time (time that the shutter is open), binning (determines the pixel size on the CCD), focus and subject height (focal plane), f/stop (aperture size) and field of view (FOV). The preset FOVs (square image regions) for the IVIS Imaging System 200 Series are 4, 6.5, 13, 20 and 25 cm (width of FOV).

Pressing Acquire or Acquire continuous photos in the IVIS System Control window initiates image and data acquisition on the IVIS. Acquisition times can range from a few seconds up to several minutes. For our experiments, we use the 60 second default exposure setting.

A Close-up image (small FOV) may provide greater resolution, but not necessarily enhanced sensitivity compared to an image taken using a larger FOV.

After the imaging procedure, animals are removed from the imaging chamber, returned to their cages and monitored until they recover from the anesthesia.

7. Data analysis

The luminescence data is analyzed using the Living Image software (Xenogen) and corresponds to the light intensity expressed as the number of photons detected by the CCD camera. These data are represented by a pseudo-color image that is overlaid on to a black and white photograph of the animals. Specific areas of the image may be analyzed by creating regions of interest (ROI). The Living Image software does provide a variety of data output options. One of the simplest ways to analyze the data is to select a region of interest (ROI) and measure the average # of photons/second that is detected. These data can then be exported to a spread sheet such as Microsoft Office Excel (Microsoft Corporation). GraphPad Prism (GraphPad Software) was subsequently used to generate graphs for this manuscript.

3. Representative Results

Summary:

IVIS technology enables the visualization of luciferase-expressing *Leishmania* spp. parasites in living anesthetized BALB/c mice in real-time. Once the substrate luciferin distributes through tissues, the rapid oxidation of D-luciferin by luciferase expressed by the transgenic parasites causes light to be emitted. The photons that are not absorbed by the tissue are detected at the surface of the animal by the CCD camera of the IVIS imaging technology.

1. Models of infection with parasites causing human visceral versus human cutaneous leishmaniasis.

Murine models of visceral leishmaniasis are more problematic than models of cutaneous leishmaniasis, since the progress of infection requires euthanasia of groups of mice at each time point assessed. A limitation of IVIS is that light emissions are quenched to different extents in different visceral tissues, and tissues with high blood flow such as liver and spleen may be more problematic than skin. Therefore, IVIS may not be as sensitive for detection of luciferase-expressing parasites in livers and spleens as in the skin. It is also advised that parasite loads should not be compared between tissue sites. Nonetheless, parasite loads in the same tissues can be compared between age-matched infected mice of the same strain.

The luciferase signal was readily detected in visceral organs in our experiments, facilitating comparison of infections between animals. An example of a 5-week infection with a luciferase-expressing parasite causing human visceral leishmaniasis, L.i . chagasi SSU/IR1SAT-LUC(A), is shown in Figure 1. After 5 weeks of infection, visceralized parasites can be detected in the livers of all mice and in the spleens of mice 1 and 2. Figure 2 shows a dose titration with the same parasite line, one hour after introduction of the parasite. An example of a longitudinal infection with parasites causing human cutaneous leishmaniasis, *L. mexicana* SSU/IR1SAT-LUC(A), is shown in Figure 3.

2. Kinetics of luciferase detection.

Preliminary kinetic studies are essential to maximize the detection of photons emitted from the bioluminescent parasites. It is important to determine this empirically, as the optimal imaging time will likely vary depending on the number and brightness of the parasite isolate and on the anatomical location of the luminescent microorganisms in mice. The kinetic studies are dependent on the rate of luciferin distribution throughout tissues, the mouse tissue harboring luciferase-expressing Leishmania, and the efficiency of luciferase expression/enzyme activity in each parasite isolate. Mice are infected and inoculated with luciferin as described above, and a sequence of images is collected every 2 minutes after luciferin injection. The magnitude of light emitted is quantified and graphed to determine the maximum detection point (see example in Figure 1B). Once the time of maximum light detection is determined, all images in the experiment should use the same time of imaging after inoculation of luciferin, as in Figure 1A. Our kinetic studies demonstrated a peak luciferase signal from the liver of mice infected i.v. with *L. i. chagasi* between 10 and 25 minutes after i.p. luciferin inoculation (Figure 1B).

3. Assessing the efficiency of parasite inoculation.

IVIS can be used to estimate the consistency and efficiency of initial infection with luciferase-expressing *Leishmania* spp. As long as the same route is used for each animal, the photons emitted can be used as a rough estimate of inoculation efficacy. An example is shown in Figure 2, in which BALB/c mice were inoculated with the indicated numbers of metacyclic *L. i. chagasi* promastigotes, a visceralizing species of Leishmania. One hour after infection mice were inoculated with luciferin i.p., and after 20 minutes images were taken. The figure shows the black and white image alone (Figure 2A) and the image overlaid with pseudo-colored luminescence (Figure 2B). Figure 2C shows the number of photons emitted from chosen regions of interest (ROI) from the mouse images.

The example in figure 2 demonstrates that the infection dose is directly proportional to the gradation of photons emitted with increasing numbers of parasites. The data show that, similar to *L. major* and *L. mexicana*, imaging of cutaneous infections with *Leishmania* spp. is very sensitive, allowing fewer than 10^4 parasites to be clearly visualized. Data such as these can be used to quantify infection intensity. In the example shown in Figure 2B, there are approximately 1 photons/sec/parasite.

4. Monitoring the course of infection in mice.

Murine models of cutaneous leishmaniasis characteristically use lesion size as a measure to follow infections longitudinally. Although a good estimate of disease progression, lesion size is affected by the degree of inflammatory response in addition to the rate of parasite replication in tissues. Mice must be euthanized at the end of the experiment to actually measure parasite load. IVIS can be used to estimate the progression of parasite loads longitudinally in cutaneous tissue of mice infected with *Leishmania* species that cause cutaneous leishmaniasis. Demonstrated in Figure 3, BALB/c mice were inoculated subcutaneously on day 0 with 10^5 *L. mexicana* expressing luciferase and imaged on the sequential weeks after infection. Figure 3A exhibits representative images at 10-31 weeks after infection. The same data are shown in quantitative form in Figure 3B. Importantly, the numbers of parasites at the infection site can be quantified by IVIS before a lesion is detectable by other methods.

Over time, the progressive increase in bioluminescence corresponds to an increase in parasite numbers. The signal strength, i.e. photons/parasite/second, can vary as a function of parasite numbers, tissue location, growth phase and parasite health in vivo. It is therefore important to calibrate the relationship between parasites and luminescence before assuming that bioluminescence is directly proportional to parasite numbers. In the case of *L. major* [15] or *L. mexicana*,[16] there appears to be relatively little attenuation of bioluminescence in amastigotes within footpad lesions relative to promastigotes. Preliminary data suggest that attenuation may be more profound for *L. chagasi infantum*[17] or for *L. braziliensis*.[15]

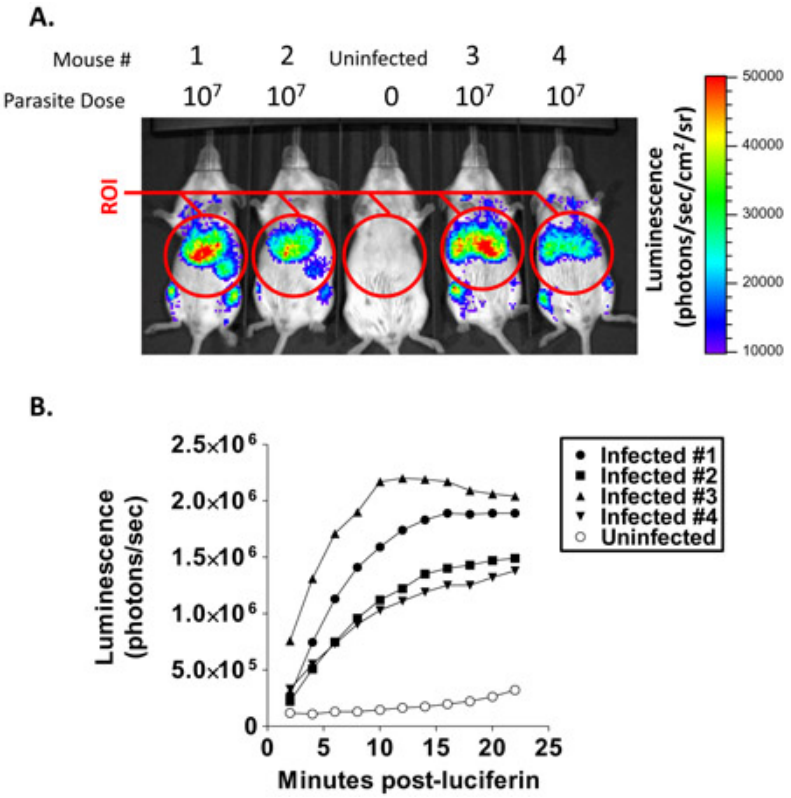

Figure 1. Kinetics of luciferase detection. Kinetic analysis of luciferin distribution was performed to establish the optimal time for imaging *Leishmania* infection in the liver. Mice were infected i.v. with 10^7 *L. i. chagasi* pIR1SAT-LUC(A). A) Four infected mice were imaged along with one uninfected mouse after 5 weeks of infection. Luciferin was injected i.p. into all five mice and images were collected every two minutes for 24 minutes. ROI were selected and luminescence was measured and quantified (B).

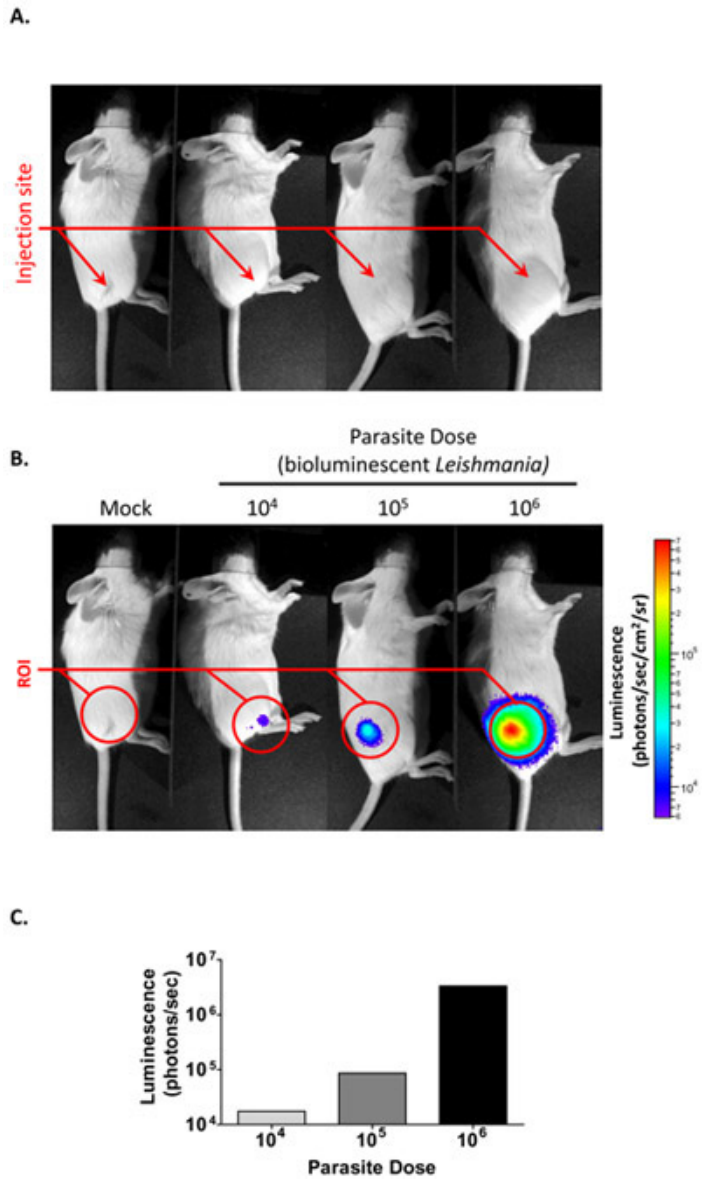

Figure 2. Assessing the efficiency of parasite inoculation. Imaging and quantifying bioluminescent *Leishmania* parasites during infection with IVIS. Wild-type BALB/c mice were injected intradermally in the lower right flank with HBSS (mock) or transgenic *Leishmania* i. chagasi *L. i. chagasi* pIR1SAT-LUC(A). The mice were then analyzed with the IVIS imaging technology one hour after inoculation. A) A black and white photograph of the mice was taken immediately before the luminescent signal (light intensity) was measured. Red arrows point to the injection site on each animal. B) The pseudo-color image of the luminescence was overlaid on the photograph. C) Regions of interest (ROI) were selected (red circles) and the luminescence was quantified in each ROI. The data represent the net luminescence of infected ROI minus the ROI of mock infected.

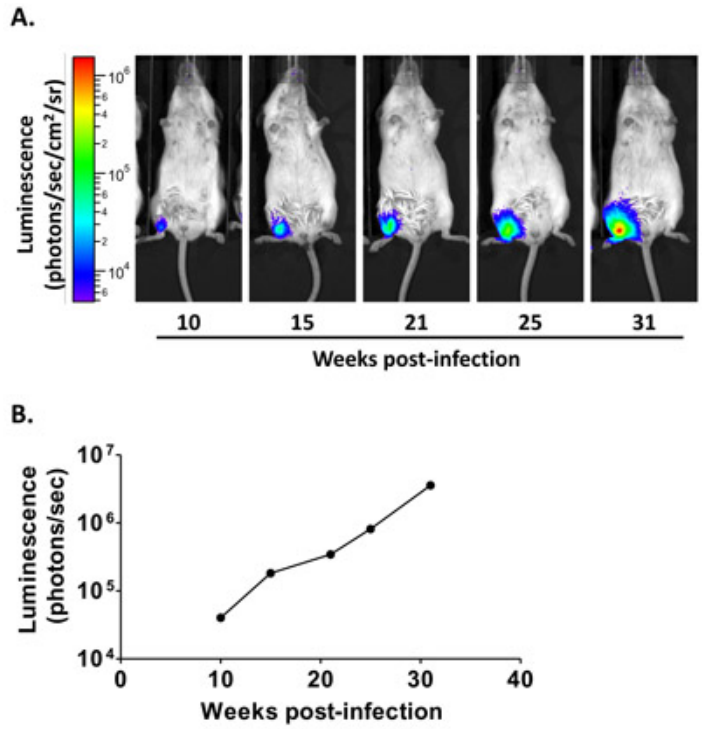

Figure 3. Long-term infection with a parasite causing human cutaneous leishmaniasis. Time course evaluation of parasitic burden in a single animal injected with bioluminescent *Leishmania* mexicana pIR1SAT-LUC(A). A wild-type BALB/c mouse was injected subcutaneously in the hock with 100,000 stationary-phase transgenic *Leishmania* mexicana pIR1SAT-LUC(A). IVIS data were collected from the infected mouse at the indicated number of weeks post-infection. A) Pseudo-colored images of the luminescence were overlaid onto black and white photographs from corresponding time points. B) The net ROI was determined by measuring luminescence in the infected hock and subtracting background ROI from the contralateral uninfected hock was graphed. The scatter plot represents net ROI at various time points post-infection.

Disclosures

No conflicts of interest declared.

Discussion

The *in vivo* imaging system (IVIS) provides a method for whole animal imaging or *in vivo* imaging experimental infection models of different forms of leishmaniasis.[18,16] The *Leishmania* spp. parasites can be engineered to express firefly luciferase at a level that is detected *in vivo* with the IVIS imaging technology. One of the major advantages of this method is that it allows non-invasive visualization of *Leishmania* spp. inside the live animal host. This method has been applied to other infectious disease models, using infectious agents that can express transgenes.[19 20] Furthermore, IVIS has been used as a model to assess drug therapy of *Leishmania major* infection in the skin of C57BL/6 mice.[16,21]

There are some important caveats and limitations of this method. The strength of the light signal detected by the CCD camera can be affected by the location of the parasites within the mammalian host, by the strength of luciferase expression in the parasites, by the availability of co-factors for the oxidation reaction of D-luciferin and by absorption of light by overlying tissues. Furthermore, the signal strength in livers and spleens is weaker at the level of photons per parasite per second than the signal in the skin, likely due to quenching by local blood supply and the overlying tissues. Quantification of the emitted photons can be used to compare progressive infection in a single animal over time, or in the same tissue between animals. [16] However until further optimized, these caveats will limit the sensitivity and thus the utility of the method for quantitative assessment of deep infections with the visceralizing *Leishmania* spp. Finally, images are likely to be weaker in black (e.g. C57BL/6) mice than in white (e.g. BALB/c) mice. Shaving the hair off skin overlying the area of interest can be helpful in detecting the signal from black mice.

Additional considerations are the need to validate the IVIS as a quantitative measure of parasite loads. Comparisons between microscopy, qPCR and IVIS have been made in a few instances, but would be needed with each system to validate the use of the method as a measure of disease progression.[22 16] New methods for imaging are emerging, which could be coupled to bioluminescence images.[16] In addition to transgenic parasites, animal hosts can be engineered to express bioluminescent or fluorescent molecules. Future studies using this technique could include animals expressing transgenes in specific cellular compartments to detect host factors involved in pathogenesis and parasites simultaneously.

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