

Full length article

Generation of stable *L. major*^{+EGFP-LUC} and simultaneous comparison between EGFP and luciferase sensitivity

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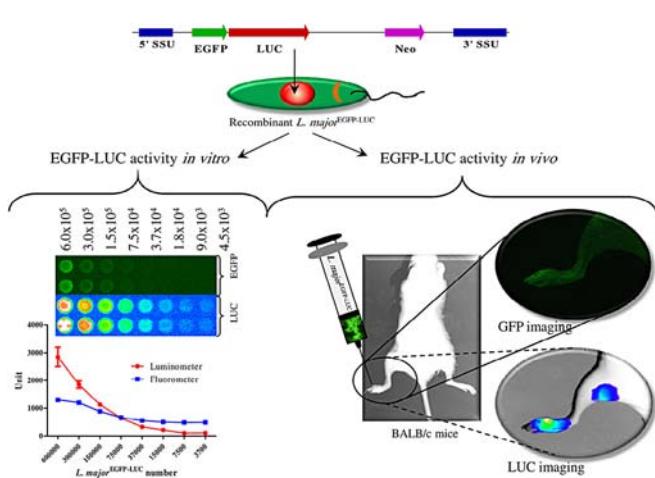
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HIGHLIGHTS

- Stable transgenic *L. major* expressing fused EGFP and firefly protein is feasible.
- Using two reporter genes simultaneously increases the experimental sensitivity.
- LUC was 10-fold more sensitive than EGFP in promastigote stage.
- EGFP and LUC are appropriate for *in vitro* and *in vivo* evaluations respectively.

GRAPHICAL ABSTRACT



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ABSTRACT

Because of the lack of an accurate and sensitive tool to evaluate the parasitemia level, treatment or prevention of leishmaniasis remains an important challenge worldwide. To monitor and track leishmanial infection by two parameters in real time, we generated stably transgenic *Leishmania* that express a bi-reporter protein as fused EGFP and firefly luciferase. Using two reporter genes (*egfp-luc*) simultaneously increases the experimental sensitivity for detection/diagnosis, and *in vitro* quantification of parasites as well as real-time infection in mice. Through different specific tools, EGFP and LUC signals from the parasite were detectable and measurable within a mammalian host and promastigotes. Here, the LUC protein provided a higher level of sensitivity than did EGFP, so that infection was detectable at an earlier stage of the disease in the footpad (injection site) and lymph nodes by bioluminescence. These results depicted that: (1) both quantitative reporter genes, EGFP and LUC, could be simultaneously used to detect parasitemia *in vitro* and *in vivo* and (2) sensitivity of firefly luciferase was 10-fold higher than that of EGFP in promastigotes.

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1. Introduction

Leishmaniasis remains one of the world's most neglected life-threatening diseases (Alvar et al., 2012). Great efforts have been made for effective drug or protective vaccine development but both remain a challenge for scientists. Many factors contribute to the slow progression of successful therapy or vaccination, the primary one being the lack of a potential tool or technique to estimate residual parasites after treatment or vaccination. Therefore, rapid, specific, and highly sensitive tests for laboratory diagnosis and research are highly in demand.

Conventionally, *in vivo* diagnosis of *Leishmania* is observational, and includes lesion size measurement, culture, limiting dilution assay or direct observation of amastigotes using specific staining (Mehta et al., 2008). Conventional techniques are laborious, take hours to days to finish with reliable results, are not real-time analysis, and are highly variable. Besides being highly technically demanding, data analysis based on the outputs of each technique is very critical. For example, in mice experiments, lesion size after parasite inoculation is not a precise reflection of parasite number because of false-positive inflammation (Mehta et al., 2008; Roy et al., 2000). Modern molecular diagnostic methods including the analysis of both DNA and RNA are also used for the assessment of infection. Also, to successfully accomplish an experiment, large number of animals should be infected and sacrificed at different time points during the study. Because of this limitation in conventional methods, each mouse cannot be monitored more than once. More importantly, in majority of the cases during the experiment, scientists have to follow the disease process in individual mice, but this is not achievable after killing the animals (Michel et al., 2011); therefore, the results of different assays could not be compared. Furthermore, small numbers of parasites are not easily and reliably detectable, and growth, multiplication, and distribution of parasites within different hosts or tissues are not equal. Response of each individual animal to a drug or antigens is different from other individuals in the same group. In other words, some mice may have faster and serious reaction than others. All these drawbacks of conventional methods have convinced the scientists to replace precise and rapid tests in real-time detection for killing of the animals (de La Llave et al., 2011; Lang et al., 2005).

Importantly, effectiveness of candidate drugs should be tested against both intracellular and extracellular forms of *Leishmania*. A tool to detect low number of *Leishmania* parasites is needed; however, the main drawback of the old methods is the possibility to detect a large number of parasites without monitoring infection in the live animals.

Today, reporter genes with their extraordinary properties help to make better interpretations of ongoing events in real-time *in vivo*. The reporter genes display distinct phenotypes that help to measure infectivity rate within cells, but different reporters show different degrees of technical sensitivity (Dube et al., 2009). EGFP and LUC are two types of optical reporter proteins that individually or fused with other peptides are used for *in vivo* studies and live-cell imaging. Fluorescent proteins have some advantages, such as stability in different conditions, sharp intensity, and long half-life. GFP is an inherently fluorescent, 27-kDa monomer that unlike other bioluminescent reporters needs no substrate or cofactor. But to mention a few drawbacks, its fluorescent intensity falls considerably when fused downstream of other proteins (Pedelacq et al., 2006), it radiates a background level as auto-fluorescence, and its interference with internal organs decreases its sensitivity (Tsien, 1998). Luciferase is a better reporter of interest, because of a higher sensitivity, lower background, and lack of auto-fluorescence. But it is still very expensive, requires luciferin as a substrate, and is ATP and O₂ dependent (Contag and Bachmann, 2002).

So far, several transgenic *Leishmania* strains expressing reporter genes on episomal plasmids (Mehta et al., 2008; Thalhofer et al.,

2010) or different genomic loci such as rRNA have been generated and used for *in vivo* monitoring of infection or drug efficacy studies (Bolhassani et al., 2011; Lang et al., 2005; Lecoeur et al., 2007; Rocha et al., 2013; Varela M et al., 2009).

In the present study, a dual-labeled *Leishmania* parasite with *egfp-luc* reporter genes was generated to further potentiate the sensitivity of diagnostic tests. The two reporter genes together could enhance the sensitivity of detection of low parasite number within cells using several instruments such as epifluorescence microscope, FACS, fluorometer, liminometer, and imaging system. Also, we focused on these parasites for studying drug efficacy against cutaneous leishmaniasis on BALB/c mice using Amphotericin B (AmB) as a model. Several parameters including footpad swelling, body weight, parasite burden, *in vivo* BLI and luminescence were measured to determine the potential of these recombinant parasites for drug screening approaches.

2. Materials and methods

2.1. Animals and parasites

Female BALB/c mice (6–8 weeks old from the Pasteur Institute of Iran) were kept under standard conditions of light and diet in the animal facility and used as an acceptable model for *L. major*.

L. major (strain MHRO/IR/75/ER) was cultured in complete liquid M199 medium (Sigma, Germany) supplemented with 5% heat-inactivated fetal calf serum (HI-FCS, Gibco, UK), 40 mM HEPES, 2 mM L-glutamine, 0.1 mM adenosine, 0.5 µg/ml hemin (all from Sigma, Germany) and 50 µg/ml Gentamicin sulfate (Biosera, France). All wild-type and recombinant parasites were cultured at 26 °C.

The transgenic parasites in this study were *L. major*^{EGFP} and *L. major*^{EGFP-LUC}. To keep the virulence; transgenic parasites in metacyclic phase were injected to mice several times. The isolated parasites from one mouse were cultured in complete M199 medium and used for re-infection. EGFP expression of all transgenic parasites in different steps of the experiment was monitored by fluorescent microscope (Nikon microscope E200) and measured by flow cytometry (BD Biosciences, excitation and emission peaks at 490 and 530 nm, respectively). For *in vitro* bone marrow-derived macrophage infectivity assay as well as mouse infections, stationary-phase promastigotes were used.

2.2. Construction of pLEXSY-EGFP-LUC recombinant vector

The plasmid expression vectors pEGFP-LUC (Biosciences Clontech) encoding EGFP (Green Fluorescent Protein, ~ 720 bp) and firefly luciferase (LUC, from firefly photinus pyralis, ~ 1650 bp) open reading frames was used to obtain a fusion gene separated by ~40 bp polylinker. The *egfp-luc* fragment was digested by *Aat*II/*Hpa*I and later inserted in *Aat*II/*Eco*RV site of pAT153 vector as intermediate plasmid. Then ~2.7 kb DNA fragment containing *egfp-luc* fusion gene was cut with *Nhe*I/*Nco*I and was cloned at the same site on pLEXSY-neo (Jena Bioscience, Germany), specific *Leishmania* expression vector. Recombinant pLEXSY-EGFP-LUC vector was subject to *Scal* and *Bgl*II enzymatic digestion and PCR with *egfp* gene specific primers to confirm both *egfp* and *Luc* gene integration.

2.3. Transfection and drug selection

Wild-type parasites were transfected by 5'SSU-*egfp-luc*-3'SSU fragment as previously described (Bolhassani et al., 2011). Briefly, promastigote wild-type (WT) parasites in mid-log-phase were harvested by centrifugation (3000 rpm, 10 min, 4 °C), washed in PBS (8 mM Na₂HPO₄, 1.75 mM KH₂PO₄, 0.25 mM KCl, 137 mM NaCl; pH 7.2) and resuspended in cold electroporation buffer (EPB: 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose; pH 7.5) (Robinson and Beverley, 2003) at a final density of 4.0 × 10⁷

parasites/ml. Four hundred microliters of parasite suspension was mixed with ~5–10 µg of large fragment of Swal digested plasmid in 50 µl total volume. The mixture was put in a 0.2 cm electroporation cuvette (Bio-Rad, USA) and stored on ice for 10 minutes. Then the parasites were electroporated twice at 450 V, 500 µF using Bio-Rad Gene Pulser Ecell (USA) device with 20 second intervals. To subdivide at least into 3 generations, electroporated cells were kept in liquid media in the absence of antibiotic for 24 h prior to selection. Then transfected parasites were centrifuged and plated onto semi-solid M199 media. One part was plated on 1% noble agar containing 50 µg/ml G418 (Geneticin, Gibco, UK) and the other part on plates without any drug as a positive control. A few single clones were then selected and cultured in liquid medium containing at least 25 µg/ml of G418. In each experiment, wild-type parasites electroporated without DNA were used as negative or mock control.

2.4. Genotyping verification

After removal of dead cells in 700 rpm/5 min, the live promastigote parasite in log phase were pelleted in 3000 rpm/10 min and washed in PBS (pH 7.2). Genomic DNA was isolated by DNeasy Blood and Tissue kit (Vivantis, USA) and was PCR amplified with several primers. Specific primers for *egfp* gene (EGFP1: 5'-AT GAT A TC A AGA TCT ATG GTG AGC AAG GGC-3' and EGFP2: 5'-GC TCT AGA TTA GGT ACC CTT GTA CAG CTC GTC-3') (Bolhassani et al., 2011) and *luc* gene (LUC F: 5'-GCT AAG CTT ATG GAA GAC GCC AAA AAC ATA AAG-3' and LUC R: 5'-ATT CTA GAT TAC ACC GCG ATC TTT CCG CCC TT-3') were used to confirm genomic integration. Also, to check accurate integration of heterogeneous genes into exactly rRNA locus of parasite, two primers has been used: forward primer from genome (F3001: 5'-GAT CTG GTT GAT TCT GCC AGT AG-3') and reverse primer from backbone of plasmid (A1715: 5'-TAT TCG TTG TCA GAT GGC GCA C-3') (Bolhassani et al., 2011). A ~1000 bp long product was expected since the sequence of A1715 primer is absent from vector backbone.

2.5. Western blotting

Western blotting analysis was performed according to previous experiment in the lab (Mizbani et al., 2010). Briefly, equal numbers of whole parasites (~1 × 10⁵ parasite) in logarithmic phase were boiled in loading buffer and electrophorized on 12.5% SDS-PAGE gel. Separated proteins were transferred onto nitrocellulose membranes (Protean, Schleicher & Schuell, Germany) and blocked in 2.5% BSA/0.1% Tween20 in TBS (20 mM Tris-HCl (Sigma) pH 7.4, 150 mM NaCl) overnight at 4 °C. Blots were incubated for 2 hours at room temperature with diluted 1:6000 monoclonal anti-GFP-HRP or 1:10,000 for anti-luciferase (Acris Antibodies GmbH) antibodies in blocking buffer (TBS, 2.5% BSA/0.1% Tween20). Unbound antibody was removed by washing. The blots were then incubated with DAB (Sigma) solution (0.05% W/V DAB powder solubilized in 50 mM Tris-HCl (pH 7.4) along with 0.01% V/V H₂O₂ (Sigma) as substrate until specific bands were developed then the reaction was stopped by H₂O. The wild-type parasite was used as negative control.

2.6. EGFP intensity and luciferase activity in promastigote

After removing dead cells, viable promastigote suspension of parasites was washed with PBS to eliminate interfering absorbance of phenol red in culture medium. To estimate the EGFP expression, 50,000 parasites per sample were flow cytometrically analyzed (Partec, Flow cytometer, Germany) and obtained data were processed by FlowJo software (TreeStar version 7.2, USA). Live gate was further used for FL1 intensity analysis of EGFP expressing parasites.

Furthermore, luminescence and fluorescence intensity together were measured by luminometer/fluorometer reader (Multimode Microplate Reader, Synergy, BioTech). To estimate the EGFP intensity, promastigote suspension of *L. major*^{EGFP-LUC} was serially diluted in PBS (ranging from 10⁵ to 10³ parasites) in black 96-well plate (Brand, Germany). The fluorescent sensitivity was adjusted to 45 for EGFP signals. To measure the luciferase activity, 50 µl of parasite suspension was serially diluted by doubling dilution in 50 µl of PBS or lysis buffer (Glo lysis buffer, Promega, USA) and in 96-well black microplates at room temperature. After 5 minutes of incubation, equal volume of luciferin (Promega) was added as substrate to each well and Luciferase activity was measured by Luminometer (1 s/well and sensitivity of 100 with top reading mode).

2.7. EGFP intensity and luciferase activity in amastigote

To measure Fluorescent intensity, B10 cell lines (bone marrow-derived MØs, was gift from Prof. Martin Olivier, McGill University, Canada) were maintained at 37 °C and 5% CO₂ in DMEM medium (Sigma) supplemented with 10% HI-FCS, 50 µg/ml Gentamicin sulfate, L-Glutamine and HEPES. The cells (2 × 10⁵ cells/well/ml) were seeded onto 24-well plates and after 24 h, were infected by stationary phase promastigotes in PBS at a MOI (multiplicity of infection) of 10:1 (parasites/cell). Free parasites were washed away 4 h later with PBS and cells were supplied by fresh medium once again. At different time points post infection (24, 48 and 72 h) cells were detached and prepared for flow cytometry analysis. All experiments were performed in duplicate for at least 3 times.

To measure luciferase activity, 4 × 10³ cells/well/100 µl were seeded onto 96-well black plates. Twenty-four hours after incubation, stationary phase promastigotes were added at a MOI of 5:1 to each well. At different time points post infection (24, 48 and 72 h), cells were disrupted by lysis buffer and luciferase activity was measured immediately with top reading mode in the presence of luciferin (50 µl/well). Before reading absorbance of luciferase activity, the media were removed from all wells. Non-infected cells were measured as a negative control to show background level.

2.8. In vivo imaging with LUC and EGFP signals

BALB/c mice were infected with metacyclic promastigote of recombinant *L. major*^{EGFP-LUC} subcutaneously. Infection progression was monitored by EGFP or luciferase signals *in situ*. Each animal was used as its own control in different time and uninfected footpad was monitored as negative control. To remove the fluorescence background level during *in vivo* monitoring, whole leg and rump of mice were epilated.

To observe the LUC signals, the substrate D-Luciferin Potassium Salt (Caliper Lifescience) was dissolved in calcium and magnesium free PBS (Dulbecco's PBS, PAA, Austria) at 15 mg/ml concentration and injected intraperitoneal, 5 min before anaesthetization. Then, mice were anaesthetized (intraperitoneally) with Ketamine (10%)–Xylazine (2%). *In vivo* imaging was done for each mouse separately using KODAK imaging system (system FX Pro). The images were captured using three modes in different exposure times: luciferase (10 min), white (1 s) and GFP (30 s). To record EGFP signaling, the black–white and color images were overlaid together. Then, the rainbow color was overlapped on light normal image. After ROI selection, number of pixel/ROI was counted to quantify light level using Molecular Imaging V.5.0.1.27 software.

2.9. Infection and treatment schedule

Four different groups of BALB/c mice (about 20–22 g each) with at least 15 mice per group were challenged by 5 × 10⁶ parasite/ml of WT (groups 1 and 2) or *L. major*^{EGFP-LUC} parasites (groups 3 and

4) subcutaneously. Groups 2 and 4 were then treated by AmB 3 weeks after challenge. Groups 1 and 3 remained untreated during experiment as control. Metric measurement of the footpad swelling was weekly performed using standard caliper reading. 160 mg/mouse/day AmB (Photericin B, Cipla, India) in DMEM was administered to treat infected BALB/c mice in groups 2 and 4 for 10 consecutive days. Every 5 days, body weight was measured and recorded. Owing to high toxicity effect AmB on mice, more mice were included in treated groups than the rest.

2.10. Parasite burden by limiting dilution

One day before and after treatment, parasite load in draining lymph node (LN) was recorded by limiting dilution (Buffet et al., 1995). Briefly, four mice of each group were randomly sacrificed to dissect and weigh the LNs. The LNs were homogenized separately in Schneider's *Drosophila* medium (Sigma) containing 5% HI-FCS and Gentamycin (100 mg/ml). Then homogenate were prepared 20 serially dilution (from 10^{-1} to 10^{-20}) and transferred in 96 well plates in duplicate. Parasite load was calculated in this formula: $-\log_{10}$ (last dilution/weight of LN). Last dilution corresponds to the relevant dilution with live detectable parasite during 10 days of monitoring. Also, the percent of parasite inhibition (PI%) was calculated in this formula: $PI\% = [1 - (A \text{ in treated group}/A \text{ in untreated})] \times 100$ (Singh et al., 2009). A is indicative of parasite burden in each different technique.

2.11. Ex vivo assessment

As mentioned above, 1 day before and 1 day after completion of the treatment lymph nodes were isolated and homogenized in Schneider's *Drosophila* medium 5%. A small part of homogenized LNs (50 µl) was centrifuged to replace media for PBS for Flow cytometry and luminometry purposes.

To measure luciferase activity by luminometry, 50 µl of homogenized LNs in PBS was diluted serially by twofold dilution using lysis buffer in 96 well plates. After 5 min incubation at room temperature, 50 µl luciferin was added to each well and the plates were immediately read first with luminometry mode and then the fluorimetry mode of reader. The instrument sensitivity for luciferase was set to 100 with top reading mode for 1 second and results were presented as relative luminescence units (RLU). Negative (non-infected LN) and positive controls (recombinant parasite) were used in each experiment for each plate. Besides, EGFP expression was evaluated by flow cytometry (Partec, Flow cytometer) and data were analyzed by FlowJo software.

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5 software. Statistical analysis was performed using the Student's *t*-test. Significant differences between samples were statistically detected with $p < 0.05$. All data are presented as the mean \pm standard.

3. Results

3.1. Generation and characterization of stable transgenic parasites expressing EGFP-LUC

The complete ORFs of the *egfp* and *luc* genes were separated from pEGFP-LUC vector, and inserted into a specific expression plasmid in *Leishmania* pLEXSY-neo. The *luc* gene was inserted translationally downstream of *egfp* gene. Fusion reporter gene *egfp-luc* contains a

linker between them that allows the cloning of any gene into MCS between the two reporter genes (Fig. 1A, a). This vector is potentially used for both transient (episomal) and stable (genome-integrated) transfactions. In this study, tandem *egfp-luc* gene sequence was integrated into rDNA locus by homologous recombination and electroporation to maintain the expression under the control of RNA Pol I promoter (percent of identity between 5'SSU and 3'SSU sequences on vector and *L. major* is 100% and 99%, respectively). Fig. 1A, a, b and c demonstrates the details of the targeting strategies.

To select for positive clones under antibiotic pressure, the G418 concentration was raised up to 200 µg/ml. All clones resistant to the elevated G418 concentration, which were shining green by fluorescent microscopy were selected (Fig. 1B). Among the selected clones, one clone with higher expression levels of EGFP was evaluated by flow cytometry (more than 98%), and selected.

For further validation, western blot analysis was performed using anti-GFP and anti-LUC antibodies that could specifically detect single or fused reporter protein bands. Fig. 1C shows the expected specific bands (89 kDa band with individual antibodies in *L. major*⁺EGFP-LUC and 27 kDa band in *L. major*⁺EGFP). No specific protein band was detected for *L. major*^{WT}. These results confirmed the *L. major*⁺EGFP-LUC generation by EGFP and LUC gene integration into the 18s rRNA locus.

To restore infectivity in transgenic parasites, they were inoculated into BALB/c mice for three consecutive time periods. For the first injection, because of low virulence, no remarkable swelling comparable with natural infection on the footpad was observed. Parasites were then isolated and checked for fluorescent intensity and luciferase expression by fluorescent microscope, flow cytometer and luminometer. The results showed that EGFP and LUC expression was stable and intensity did not decrease after several rounds of passages in the animals (at least six times). Promastigote form of parasite maintained high levels of EGFP expression in logarithmic phase (Fig. 1D), but, in stationary phase this value decreased about 70–80% (Fig. 1E).

3.2. EGFP and luciferase activity measurements in promastigotes

We determined the activity levels of the two reporter genes in recombinant promastigotes using different systems to simultaneously measure the sensitivities of both genes.

Fig. 2 shows the results of EGFP and luciferase activity measurements in the promastigote stage. A linear relationship was observed between the number of parasites and fluorescence or luciferase expression. It was clearly demonstrated that cells diluted in lysis buffer showed higher luciferase activity than intact cells diluted in PBS alone (data not shown).

Direct comparison between the two reporter genes showed a significant difference in sensitivity. The sensitivity of detection was 10-fold higher for luciferase. The weakest fluorescence signal was detected for approximately 10,000 parasites, and LUC signal for approximately 1000 parasites (Fig. 2). We did not differentiate between different wells containing different number of parasites by fluorometer, except for concentrated wells where the number of parasites was at least more than approximately 10^5 /well. Scanning of the plate with two modes, GFP and LUC showed no detectable signal when the number of promastigotes was very low, and diluted more than approximately 10^4 parasites (Fig. 2). However, comparison of luminescence and fluorescence modes of detection indicated a good correlation between parasite burdens and fluorescence as determined by microscopy and flow cytometry.

Several transfected promastigotes were tested for EGFP expression by flow cytometry. Our data showed that the level of EGFP expression in logarithmic phase was about 91–98% (Fig. 1D).

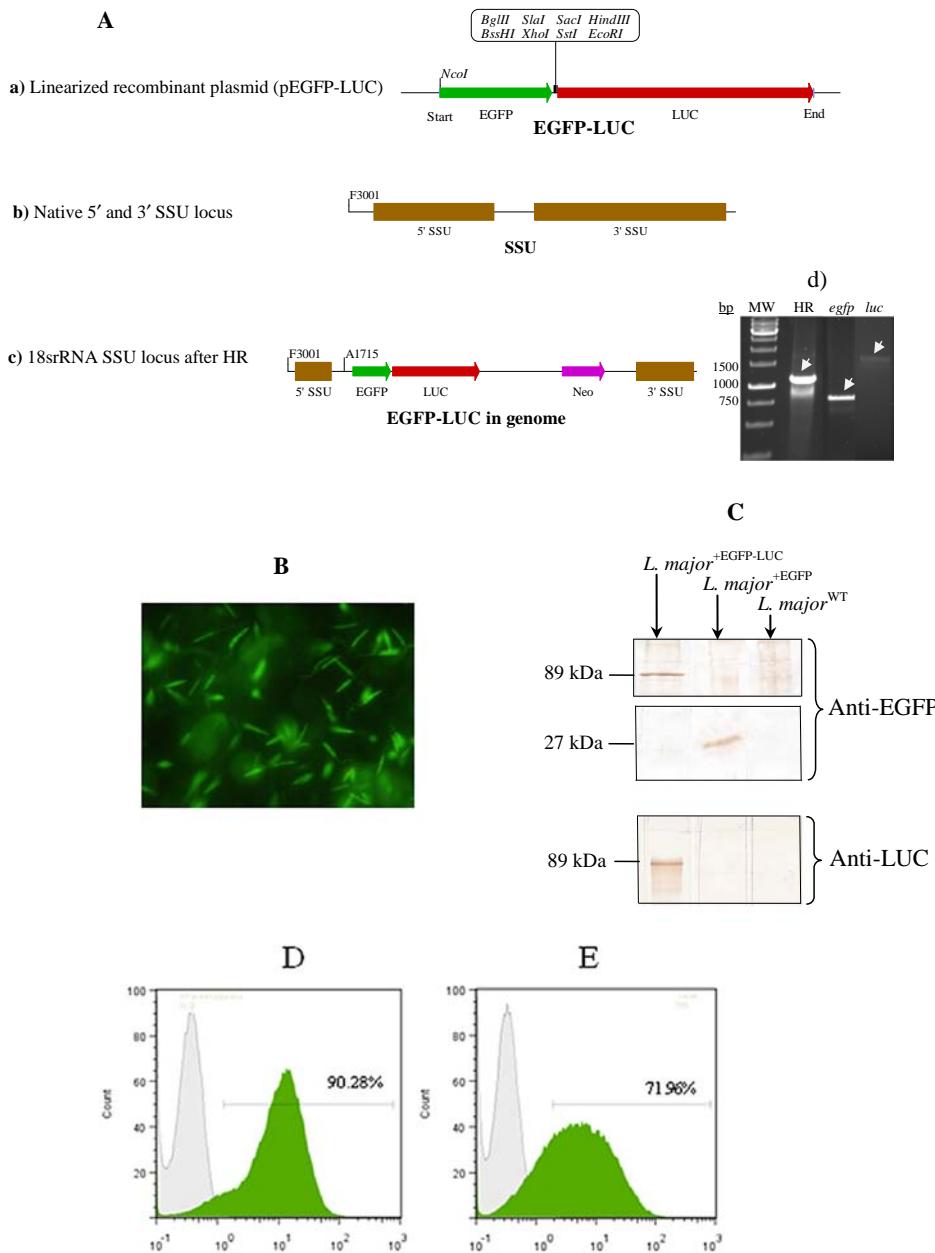


Fig. 1. Generation of stable *L. major*^{+EGFP-LUC}. (A) Schematic representation of *egfp-luc* gene into vector and SSU ribosomal DNA locus. (a) Position of the start (ATG) and stop codon (TAA) on *egfp-luc* fusion gene. (b) Position of 5'SSU, 3'SSU and matched primers on native SSU 18srRNA locus. (c) SSU 18srRNA locus after homologous recombination. The sites of restriction enzyme and primers are shown on the map. (d) Corresponding PCR products for genomic integration of *egfp* and *luc* gene into rRNA locus. Line 1, MW; line 2, SSU PCR with F3001/A1715 primers (~1000 bp); line 3, *egfp* gene with EGFP1/EGFP2 primers (~720 bp), line 4, *luc* gene with LUCF/LUCR primers (~1650 bp). Arrows indicate the expected bands. (B) Live promastigotes of *L. major*^{+EGFP-LUC} under epifluorescent microscope (100x magnification). (C) Western blot analysis using anti-EGFP and anti-luciferase specific antibodies. The molecular weight of EGFP-LUC fused protein in recombinant *L. major*^{+EGFP-LUC} parasite was ~89 kDa and for EGFP alone (in *L. major*^{+EGFP}) was ~27 kDa (positive control). Wild-type *L. major* was used as negative control. The horizontal bars indicate the expected bands. (D and E) Level of EGFP expression parasites in logarithmic (D) and in stationary (E) phase. In both the gray histogram represents wild-type *L. major* as negative control. A high expression level of EGFP were observed in logarithmic phase (~90%) but was decreased in stationary phases (~70%) of *L. major*^{+EGFP-LUC}.

3.3. EGFP and luciferase activity measurements in amastigotes

To verify whether the transfectants remain infectious, and to study the expression of EGFP and LUC fused proteins in the amastigote form, B10R cells were infected with stationary phase promastigotes of *L. major*^{+EGFP-LUC} parasite at a MOI of 5, 10 or 20 at three different time points (24, 48 and 72 h). As expected, the highest EGFP-LUC expression level was observed at 24 h with MOI of 20 by luminometry (Fig. 3A). In contrast, fluorometry did not show any fluorescence. No considerable difference between the different MOIs

was observed after 72 h (Fig. 3B). However, luminometry results showed that the recombinant parasites remained infectious to macrophages.

The fluorescence intensity of intracellular stage of parasite was monitored at different time intervals by flow cytometry. Our data demonstrated that the highest intensity was at 24 hours post infection (14.5%), followed by 8.8% at 48 hours and finally 1.12% at 72 hours post infection (Fig. 3C). It is worth to mention that there were no significant differences between cell to parasite ratios (1:5 and 1:10) infectivity in respect to EGFP expression.

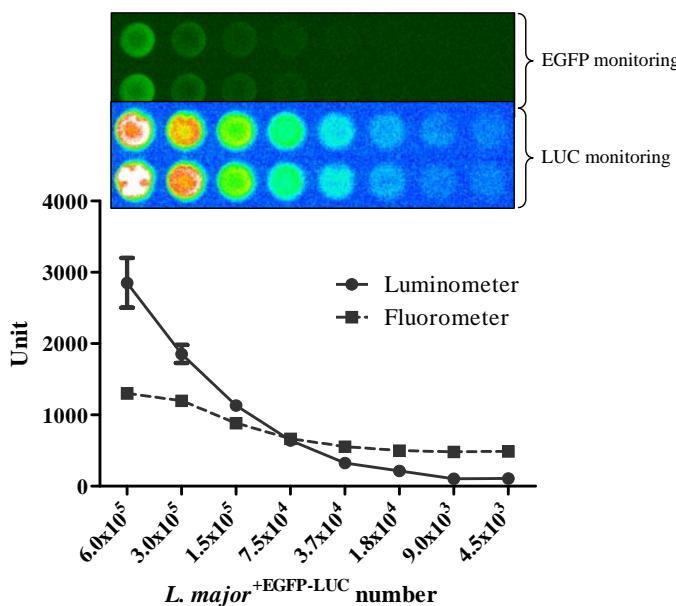


Fig. 2. Measurement of EGFP and Luciferase activity in promastigote stage. The promastigote suspension of $L. major^{+EGFP-LUC}$ was diluted serially in PBS (for EGFP) or lysis buffer (for LUC) ranging from 6×10^5 to 4×10^3 parasites into a black 96-well microplate. After 5 min, luciferase substrate was added on lysed cells and derived light was measured first by luminometry mode and then fluorometry mode. The different numbers of parasite were quantitatively compared with two different modes on: luminometer and fluorometer. Parasites were diluted serially in 96 well plates and the relative intensity values were analyzed on a graph. The bars represent the mean of triplicate wells with at least two repeated assay. The same plates were qualitatively analyzed by KODAK imaging system (system FX Pro). Data analyses confirm that the sensitivity detection was 10^3 for luciferase mode and 10^4 for EGFP mode.

3.4. In vivo monitoring of parasite development in live mouse

Here, we tried to follow the development of swelling after $L. major^{+EGFP-LUC}$ inoculation into live BALB/c mice by *in vivo* fluorescence and bioluminescence imaging systems. The mice were subcutaneously infected with transgenic parasites, and simultaneously monitored by EGFP and LUC signals at different time points. To optimize imaging and the suitable time point for injection of luciferin (i.p.) or anesthetic (i.p.), we tested different conditions. First, we monitored infected footpad of anesthetized mouse for EGFP signaling before and after injection of luciferin 7 weeks after infection. There was no difference in signaling and intensity of EGFP in both conditions. However, peritoneal inoculation of luciferin (almost limb position) showed an unexpected false-positive signal at the injection site for a short time period (5–10 min) after injection. After 24 h, we did not observe any false-positive signal of EGFP, because of distribution of luciferin into the body, which indicated that luciferin had no effect on EGFP, if injected into the opposite side of parasite inoculation (Fig. 4A).

Second, to monitor luciferase signaling, we anesthetized the mice and injected luciferin substrate at the indicated time points. Based on other reports, sensitivity of luciferin to protease and the short half-life of its activity, we had to optimize the best condition and determine the suitable time point for injection of anesthetic and luciferin. We used two infected mice 8 weeks after infection for optimization of luciferase signal (Fig. 4B). The optimal condition was to euthanize the mice 5 min after luciferin injection. The highest level of LUC activity was detected after 45 min (Fig. 4C). Since D-luciferin distributes quickly and easily throughout the animal body, the mouse is quite ready for imaging right after i.p. inoculation of substrate. Based on the dynamic results of luciferase; we selected

10 min exposure time, 10 min after using substrate as the best condition for the next experiment.

Next, we examined the parasite propagation by fluorescence and bioluminescence imaging at the inoculation site and draining lymph nodes of four infected mice. Bioluminescence signal was detectable after inoculation of recombinant parasite in the footpad during the first hour of infectivity because of local parasite aggregation. One day later, the bioluminescence signals had disappeared. Positive infection was actually detectable at least 10 and 30 days after challenge in the footpad and draining lymph node, respectively, through rainbow imaging. Fig. 5A illustrates the monitoring results after 15 days of infection. Meanwhile, fluorescence imaging revealed infection 60 days after challenge. While mice infected with $L. major^{+EGFP}$ showed infection in the footpad at least 30 days post-infection (Fig. 5B). These data were in close agreement with a previous report (Bolhassani et al., 2011). Parallel comparison of two reporter genes in the same tissue at the same time showed that although both reporter genes are fully expressed *in vivo*, bioluminescence imaging is more sensitive and detects the infection faster than EGFP. However, we could not detect any signal from both reporter genes in the spleen (data not shown).

3.5. Assessment of drug efficacy (or generally assessment of disease)

Weekly scheduled measurement of local inflammation and lesion development and also body weight recording every 5 days, were used as standard methods to evaluate the efficacy of AmB. As shown in Fig. 6, footpad swelling in all groups was progressive due to parasite amplification but better controlled in treated groups compared with relevant control groups. However the difference was not statistically significant.

Body weight was used as a marker of toxicity. Measured before and during treatment schedule and presented as group average, a dramatic body weight loss was evident early after starting the drug, then it gradually adapted to the drug and after finishing the full dose, it was back to normal level (data not shown). Differences in footpad size between untreated groups were due to size of mice and not parasite type.

3.6. Estimating parasite load using limiting dilution

We had to measure parasite burden at different time points before and after treatment. To minimum of variation in each time, 4 mice per group were sacrificed. Parasite load was significantly lower in treated groups than untreated groups as expected ($p < 0.05$) (Fig. 7B). Parasite load of untreated groups was higher compared with time preceding treatment start up. Observed difference between infected groups with wild-type and transgenic is due to variation in weight of mice. Lymph node weight records also confirmed the results coming from limiting dilution since treated LNs showed dramatic drop down in weight compared with untreated (Fig. 7A). This result was significant and in consistence with body weight to prove drug toxicity.

3.7. Ex vivo evaluation of drug efficacy by flow cytometry and luminometry

To ex vivo evaluate of drug effect between two groups treated and not, we analyzed the difference between homogenized LN of infected mice just with recombinant parasite which express reporter genes. All lymph nodes individually were analyzed by both methods. Flow cytometry analysis confirmed the decrease of parasitemia in LNs of treated group (0.963 ± 0.198). Percentage of EGFP

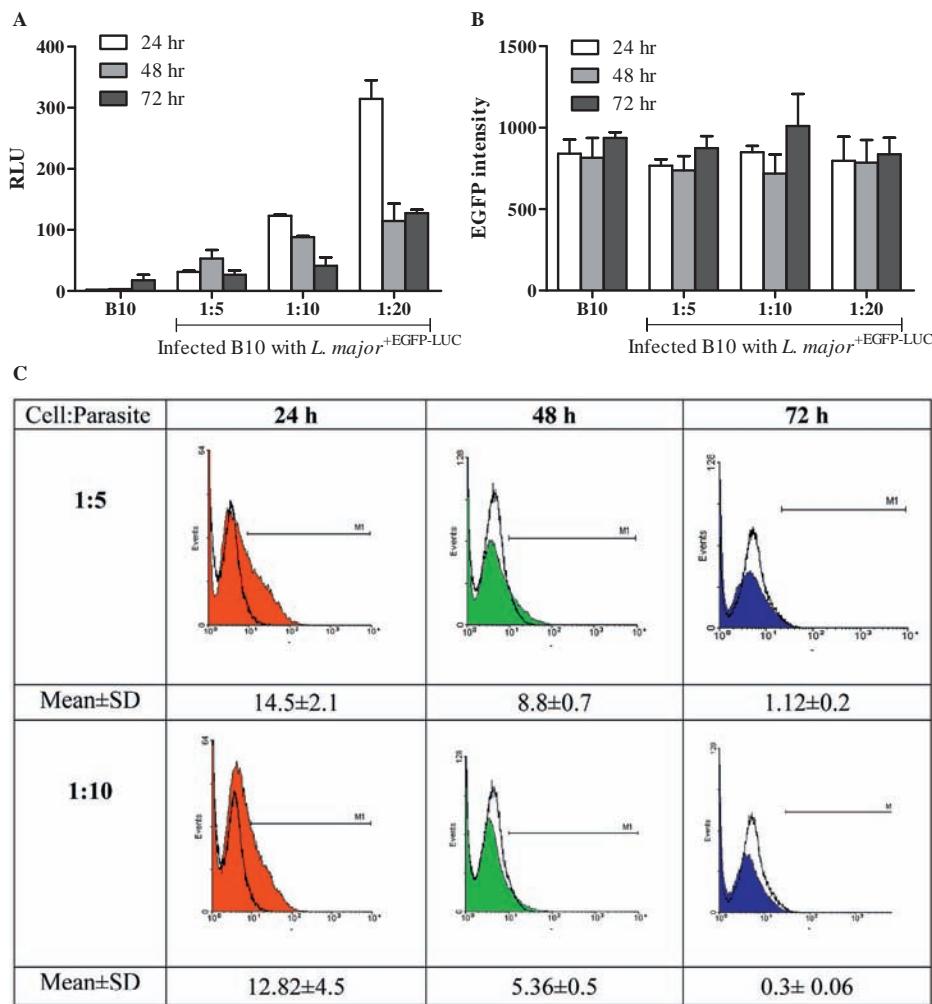


Fig. 3. Measurement of luciferase and EGFP activity in amastigote stage: The B10R cells (immortalized murine bone marrow-derived) were infected with stationary phase promastigotes of *L. major*^{+EGFP-LUC} parasite (MOI 5, 10 and 20). At different time periods (24, 48 and 72 h), EGFP expression and luciferase activity were measured by fluorometer, flow cytometry and luminometer. (A) The highest EGFP-LUC expression level was observed at 24 h with MOI 20 using with luminometer. (B) Fluorimeter did not show sufficient sensitivity to show fluorescence intensity. (C) Overlaid histograms of one clone of transgenic *L. major*^{+EGFP-LUC} and wild-type lines.

intensity was much lower from untreated (2.048 ± 0.389), although this difference was not significant ($p = 0.057$) (Fig. 8A).

In addition, emitted light derived from luciferase-luciferin reaction was quantified in LN from mice infected with recombinant parasite. A huge decrease was observed in treated mice with AmB that indicated the presence of a low number of parasites in LNs (Fig. 8B). Parasite amplification inhibition was better demonstrated by luciferase protein activity than EGFP fluorescence.

3.8. Evaluation of drug efficacy by *in vivo* imaging

To assess the drug efficacy in controlling infection, live imaging technique was used on footpad and lymph nodes of infected mice. This way there was no need to sacrifice the animal. This technique was just applicable to mice infected with recombinant *L. major*^{+EGFP-LUC}. Three weeks after challenge, 10 mice of each group were randomly selected and imaged the day after treatment was terminated. Each mouse was prepared and placed on chamber individually.

The results of BLI imaging after end course of drug therapy was presented in both treated (Fig. 9A, a) and untreated (Fig. 9A, b) groups. As shown, the luciferase activity was detectable in the lymph nodes of three mice out of 10 in treated group and seven mice out

of nine in untreated group 4 (30% vs. 80% respectively). This was a marker of parasite propagation in LN due to less effectively controlled disease. The highest level of expression of both reporter proteins was detectable only in footpad and LN, but not in liver and spleen.

Total luciferase activity in footpad for untreated group 4 was about 3.6 fold higher than treated group 3 (673394.8 ± 271011.4 vs. 361255.7 ± 213393 respectively). The difference between two groups was statistically significant (Fig. 9B). This was in good concordance with our previous observations. However no significant difference was observed at this time point by *in vivo* fluorescence imaging (Fig. 9C).

To determine the efficacy of coupled reporter gene system in infection follow up, percentage of parasite inhibition (PI%) was measured and compared between different detection methods based on parasite number or expression of reporter protein. As shown in Table 1, PI% is compared by footpad thickness measurement and parasite burden via limiting dilution for both treated groups of mice (WT or *L. major*^{+EGFP-LUC} infected) and parasite burden through flow cytometry, *ex vivo* luciferase activity and bioluminescence imaging for mice infected with *L. major*^{+EGFP-LUC}. Recombinant *L. major*^{+EGFP-LUC} is evidently more sensitive to estimate infectivity rate in mice than wild-type parasite.

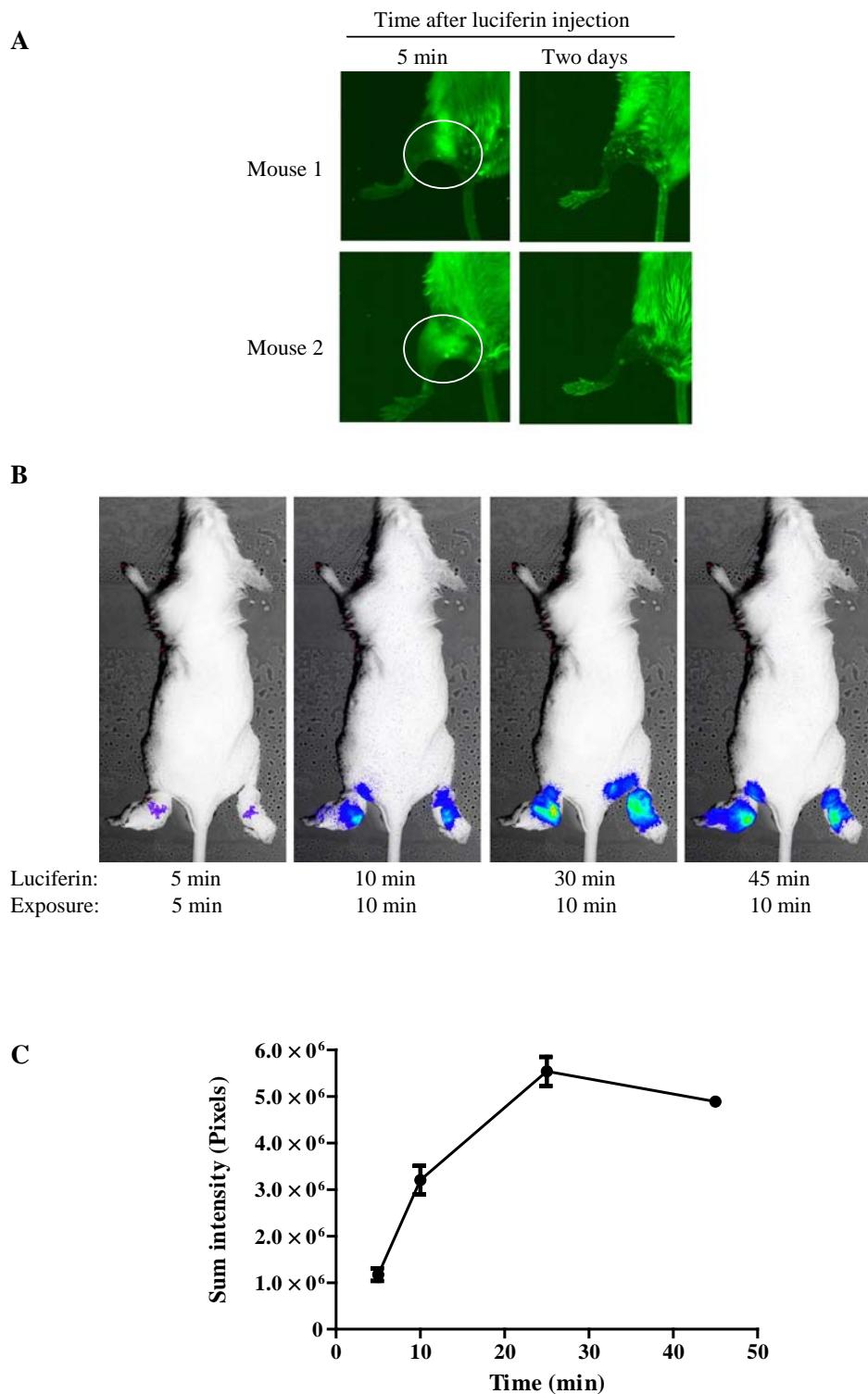


Fig. 4. Optimization of imaging condition. To optimize injection time of luciferin and anesthetic solution, different conditions were monitored on infected BALB/c mice (s.c.) by Kodak IVIS 400 system. (A) Two mice were injected by D-luciferin (i.p.) and were monitored for EGFP signaling in different time points. Left: fluorescence imaging showed background levels of fluorescence on the skin of mice while lesion size increased without any signal or a weak signal for EGFP in footpad and LN (left). White circles illustrate site of derived background from D-luciferin in early time before absorption. Right: same mice were monitored after 24 h, without any background. (B) Seven weeks post infection; images were captured in various time points after administration of D-luciferin (30 mg/ml) as substrate with different exposure times. First weak light emissions from expressed luciferase can be detected at least 5 min after injection of the substrate. The first signal of luminescence was detectable after 5 min in footpad and after 10 min in lymph node respectively. (C) Intensity rate of LUC in different time points post injection of substrate and exposure time. Twenty-five minutes after luciferin injection and exposure time 10 min were selected as the best condition to monitoring high number of animals.

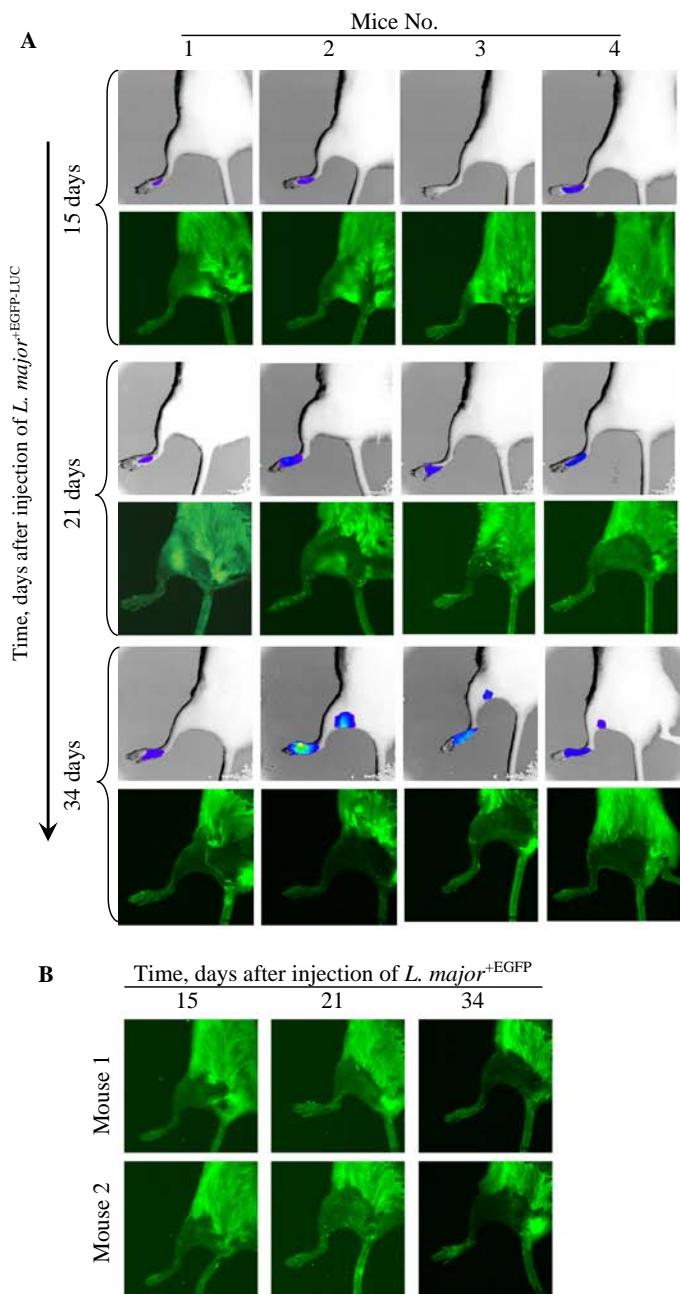


Fig. 5. Simultaneous comparison between EGFP and luciferase sensitivity in *in vivo* imaging. (A) Disease progression was monitored by *in vivo* fluorescence and bioluminescence imaging in infected mice with *L. major*^{+EGFP-LUC} subcutaneously. The rainbow image by bioluminescence was visible in mice at least 10 days after footpad challenge inoculation, while in fluorescence imaging, infection was observable at 60 days after infection. But infected mice with *L. major*^{+EGFP} showed infection in the footpad earlier (at 30 days). (B) Monitoring infectivity by *in vivo* fluorescence imaging infected mice with *L. major*^{+EGFP} during different days post infection. First signal of EGFP was detectable from at least 30 days after infection.

4. Discussion

Undoubtedly, diagnosis of *Leishmania* infection at early stages of the disease with least parasitemia level is very critical in animal research. In general, footpad swelling is used to estimate the infectivity rate in mice. However, footpad lesion size is not always directly correlated with parasite number (Roy et al., 2000).

Different imaging systems have been established so far to utilize two classes of optical reporter genes, *egfp* and *luc*. This technology

has also extensively influenced *Leishmania* studies. Different strains of *Leishmania* have been generated using knock-in strategy with reporters alone or in combination with another gene to be used as models for understanding the complexities of the parasite, diagnosis of the disease, and therapeutic objectives.

Several reports have used a combination of different reporter genes. Using GFP-LUC hybrid, tissue-specific expression of *fyuA* and *hemR* genes in BALB/c mice has been studied (Jacobi et al., 2001). Also, transgenic *P. berghei* parasite harboring luciferase-GFP fusion protein free from a drug-selectable marker has been used for drug screening (Ploemen et al., 2009).

Biological studies or identification of the role of a protein where localization of a native protein is not clear are some other applications of reporter genes. For instance, physiological role of *L. donovani* Flabarin (flagellar protein) was unraveled by fusion making of this protein with EGFP or Red Fluorescent Protein (RFP) in C- or N-terminal (Lefebvre et al., 2013).

During the past decades, several recombinant species of *Leishmania* expressing luciferase or EGFP have been used for distinct aims. Many reports have shown that viability and parasite load are directly related to the intensity of reporter genes (Latorre-Esteves et al., 2010; Roy et al., 2000; Thalhofer et al., 2010). To have a powerful tool for rapid and precise detection of the parasite during the early stage of infection, a mutant form of *L. major* expressing EGFP-LUC fusion was generated. The construct has the ability to accommodate any foreign gene in between the two reporters. Dual functionality of these parasites makes them an invaluable tool to perform several simultaneous experiments with compatible equipments such as *in vivo* imaging, *in vitro* microscopy, flow cytometry and luminometry.

The main advantage of these parasites is that they are detectable with a wide range of available instruments in each laboratory, because most laboratories in poor countries do not have many tools.

High background and unwanted effects in cells are two hallmarks of GFP. Another limitation of GFP is high sensitivity to natural and perfect folding of protein, especially when it is fused with another protein. Hence fluorescent intensity of GFP alone is very strong as compared with fusion with another protein (Pedelacq et al., 2006). Still, there are some challenges of using reporter genes. Other researchers had combined more than one reporter gene in one construct, but they observed a weak or loss of signal for some of the

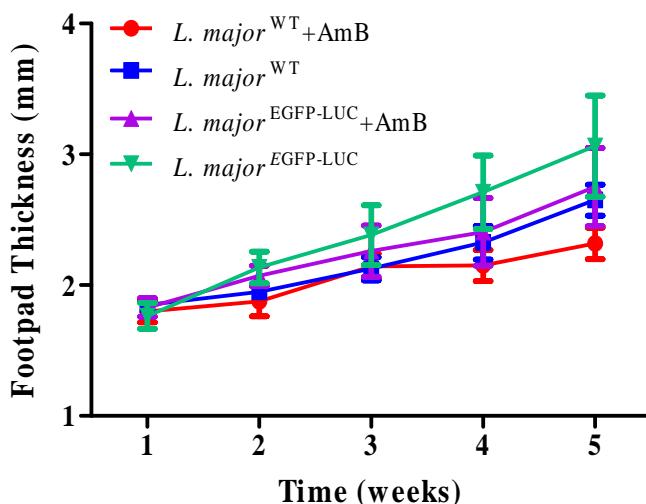


Fig. 6. Monitoring footpad. Footpad swelling was measured weekly by caliper. Two drug receiving groups showed a milder progression in footpad thickness compared with untreated groups. This difference was not evaluated as significant. The bars indicate standard deviation (SD) of for four mice in each group.

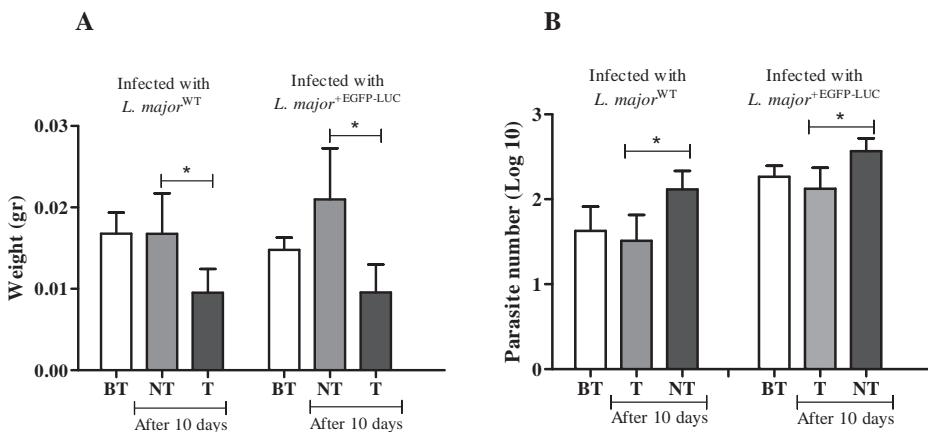


Fig. 7. AmB effect on parasite load and lymph node weight. (A) LNs were dissected out from mice corps before and after therapy. The difference between LN weights from treated and untreated groups were statistically significant ($p < 0.05$). The bars reflect standard deviation (SD) of four individual mice in each group. (B) Parasite burden was evaluated before starting the therapy and 10 days after. Parasite load of AmB treated groups was significantly lower than untreated groups or before ($p < 0.05$). The bars indicate standard deviation (SD) of LNs from four individual mice in each group. BT: Before treatment, NT: No treated, T: Treated.

components (Dubey, 2012). Sensitivity of some instruments is variable for different colorimetric proteins. For example, fluorimeter is sensitive for RFP, and it cannot read contrast GFP (Rocha et al., 2013). This could be another limitation for GFP. In this experiment, we had similar observation for measurement of GFP expression of recombinant parasites in both promastigote and amastigote forms in macrophages using a fluorimeter.

In contrast, because of the short half-lives of luciferase and luciferin, this protein showed at least background luminescence. As noted by others, *in vivo* bioluminescence is more sensitive than *in vivo* fluorescence because of the absence of auto-bioluminescence and low background activity (Savellano et al., 2010; Serganova and Blasberg, 2005). According to some reports, because of the differences in the conditions of *in vivo* enzymes, *in vivo* luminescence levels in different tissues are not directly related to their luciferase contents. It depends on tissue absorption and location, which should be calibrated before any experiment (Bloquel et al., 2006). Another limitation of optical reporter genes is the low potential for detecting parasites in deep tissues that make it impossible to estimate parasite number in whole body (Thalhofer et al., 2010).

Previously, parasitemia was detectable in infected lymph node with EGFP transfected *L. major* at day 66 after infection with stable expression system (Bolhassani et al., 2011) or at day 70 day with

episomal system (Mehta et al., 2008), which indicates there is no difference between episomal and stable expression.

According to different reports, comparison between the two imaging systems showed that both reporter genes are able to quantify, but luciferase is more sensitive because of any background derived from auto-signal. Actually this is due to luciferase activity which depends on metabolic processes through consuming the oxygen and ATP of active cell (Savellano et al., 2010).

But now the parasites have a new phenotype that helps in their identification. We could confirm two reporter genes for parasite determination per animal. Simultaneous comparison of EGFP and LUC expression showed that luciferase is more sensitive and specific as compared with EGFP for detection and quantification of parasites within infected tissue at an early stage of infection.

GFP fluorescence is very strong, and it is possible to observe this protein with fluorescent microscopy, which is a normal tool in most laboratories. In addition, GFP intensity is easily detectable *in vitro* using flow cytometry. GFP is a cost-effective protein to study *in vitro*. In contrast, luciferase is more expensive but very sensitive for *in vivo* evaluation.

However, expression of EGFP is very useful to select positive clones among a lot of cells where resistance marker genes are a barrier to drug screening (Monte-Alegre et al., 2006). For instance,

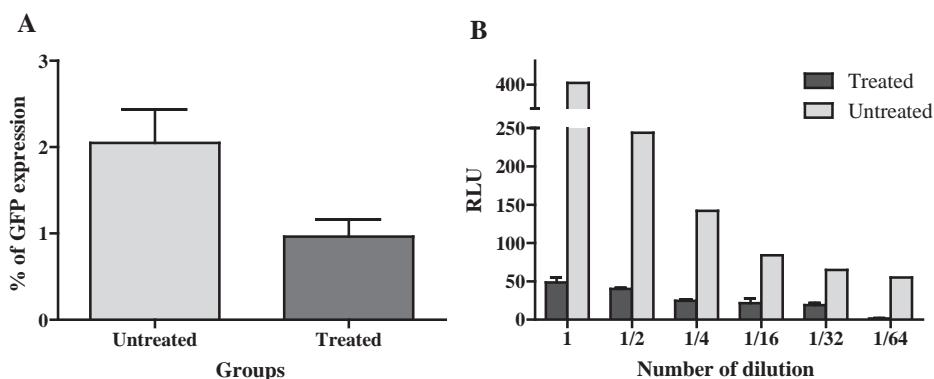
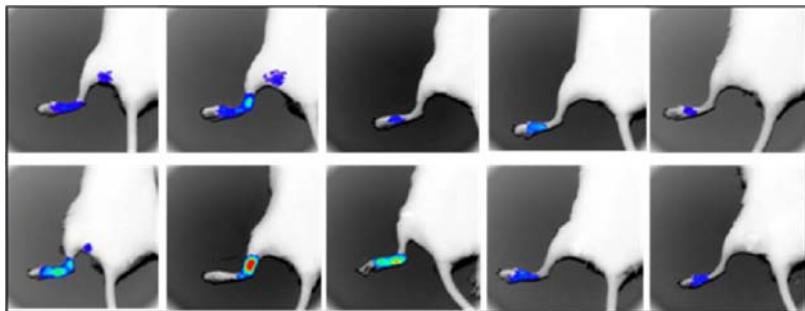


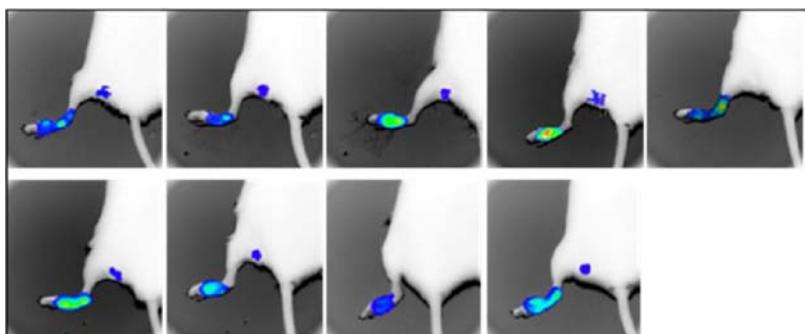
Fig. 8. The results of ex vivo analysis of parasite propagation in mice infected with *L. major*^{+EGFP-LUC}. The lymph nodes were homogenized individually and a small volume of homogenized tissue was re-suspended in PBS for flow cytometry or in lysis solution to be used in luminometry. (A) Percentage of EGFP positivity has been obtained by flow cytometry in LNs. EGFP positivity between two groups was not significant. (B) The LNs suspension was serially diluted by twofold dilution in lysis buffer and after 5 min D-luciferin was added to each different dilution. Enzymatic reaction was quantified by 1 s/100 sensitivity mode right after. Data are shown as the mean \pm SD of LUC activity of each dilution in duplicate. The results showed a significant difference between treated and untreated groups of mice infected by *L. major*^{+EGFP-LUC} ($p < 0.05$).

A

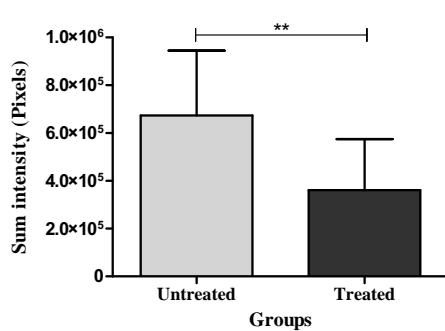
a) Treated mice with amphotericin B.



b) Untreated mice.

**B**

Imaging with Luciferase

**C**

Imaging with GFP

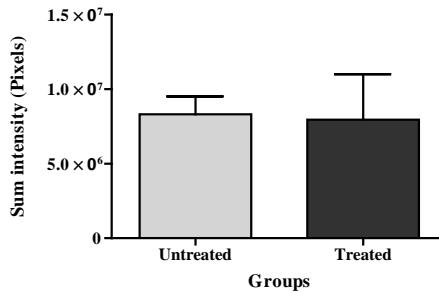


Fig. 9. Appraisal of *in vivo* drug efficacy by imaging. Bioluminescence and fluorescence imaging has been done 1 day after finishing AmB therapy. Three weeks after challenge, the mice of group 3 received amphotericin B for 10 days. Next day after finishing therapy course, BLI was performed for 10 mice of groups 3 and 4. We focused on footpad and LN in infected side of body. (A) For bioluminescence imaging (BLI), each mouse received D-luciferin shots intraperitoneally (15 mg/ml), was anaesthetized with ketamine/xylazine mixture and was placed on chamber room. Emitted light from mice was captured with rainbow color and overlaid on a white/black image. (a) Treated mice and (b) untreated mice. (B) Regions of interest (ROI) were quantified as pixels. The results are presented by sum of intensity (mean \pm SD). The difference between treated and untreated group was detected significant ($p < 0.05$). The bars reflect standard deviation (SD) of 10 individual mice in each group. (C) For fluorescence imaging, fluorescence light of individual mice was captured and ROI was quantified as pixels after BLI without changing the mouse position (fluorescent imaging data were not shown). The results are presented by sum of intensity (mean \pm SD). The bars show standard deviation (SD) of between individual mice in each group.

transfected *P. berghei* parasite (*PbGFP-Luc_{con}*) that was free of a drug-resistance marker was selected by FACS sorting immediately after the transfection process (Ploemen et al., 2009). Also EGFP intensity is particularly useful to isolate interested cells by flow cytometry in tumors (Feng et al., 2011).

As previously proven, GFP protein is very sensitive for its correct folding (Chang et al., 2005). Hence, its intensity and brightness is much lower when fused with other protein which might be the result of misfolding (Chang et al., 2005; Pedelacq et al., 2006). The main concern for using reporter genes is their cytotoxicity, which should

be addressed. Some studies have demonstrated that GFP produces free radicals that are strong cytotoxic components (Choy et al., 2003a, 2003b). However, the fate of transgenic parasites in cells or mice is unclear.

Important pending questions include effect of the reporter genes on the immune response of mice, and interaction between the two reporter genes. Also, evaluation of infectivity or immunogenicity effects of these reporter genes remains to be determined.

In summary, dual reporter expression is an excellent tool for evaluation of novel therapeutics and vaccine development. We

Table 1

Parasite inhibition is calculated based on different methods used in this experiment at week 5 after challenge for footpad thickness or 1 day after treatment for other techniques in both treated groups. The percent of parasite inhibition (PI%) was calculated in this formula: PI% = [1-(A in treated group/A in untreated)] × 100.

Parasite type	PI% in 5 weeks after challenge based on:				
	FP ^a	PB ^b	FC ^c	LUC ^d	BLI ^e
<i>L. major</i> ^{WT}	6.58%	28.57%	–	–	–
<i>L. major</i> ^{EGFP-LUC}	17.36%	17.15%	52.7%	87.9%	46.35%

^a FT, footpad measurement; A is thickness in mm.

^b PB, parasite burden; A is parasite burden.

^c FC, flow cytometry; A is percent of EGFP positive population.

^d LUC, luciferase activity; A is relative luciferase unit (RLU).

^e BLI, bioluminescence imaging , A is pixel count in region of interest (ROI).

recommend EGFP for *in vitro* evaluation and LUC for *in vivo* analysis. Thus, a combination of these two genes will help to eliminate the limitations, and strengthen the approach to *Leishmania* study.

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Conflict of interest

The authors declare that there is no conflict of interest.

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