Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We appreciate all comments made by the editor. We revised spelling and grammar issues in this revised version of the manuscript.

2. Please make the title concise to reflect the protocol being presented.

Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence and provide a systemic view of the host-parasite interaction

As requested, we changed the title to "Murine *in vivo* infection with *Leishmania amazonensis* is a reliable method to evaluate the parasite virulence within the host".

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

As requested, we modified the summary (lines 25-27 of the revised marked version).

4. Please include a single line space between each step, substep and notes in the protocol section.

As requested, we included a single line space between each step, sub-step and notes in the protocol section.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Ficoll, Corning, Durham, USA, Mitutoyo, Japan, GraphPrism Software, M199-pro, Thomas Scientific, Parafilm (Bemis, Neenah, WI, USA), Gibco, Grand Island NY, USA, etc.

As requested, we removed all commercial language from the manuscript. All commercial products were added in the revised Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

As requested, we revised the manuscript and all text in the protocol section is written in the imperative tense in the revised manuscript.

7. The Protocol should contain only action items that direct the reader to do something.

As requested, we revised the manuscript and the protocol section contain only action items.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that the Protocol contains only 2-3 actions per step and a maximum of 4 sentences per step.

As requested, we revised the protocol section ensuring only 2-3 actions per step.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

As requested, we revised the manuscript ensuring we answer the “how” question in each step.

10. 1.2: Do you check the O.D in this case? How do you ensure the logarithmic growth phase?

The logarithmic growth phase of the promastigotes´ culture is determined counting the culture daily. This is a well-established practice in any lab working with *Leishmania* cultures. The references regarding this were added in the protocol section 1.

11. In case of 1.1 the culture is grown at 25 degrees, in 1.2 and1.3 it is grown at 34 degrees? Please include the significance of the same.

The temperatures for cultivation of the 2 different forms of *Leishmania amazonensis* are well established, and these temperatures mimic the temperatures found by the parasite in the vertebrate (34ºC) and invertebrate (25ºC) hosts. So, step 1.1 (1.2 of the new version) refers to the promastigotes´ culture, which is kept at 25°C, and steps 1.2 and 1.3 (1.5-1.7 of the new version) refers to the amastigotes´ culture, which is kept at 34°C. References 37 and 38 (line 120 of the revised manuscript) substantiates this matter.

12. 2.3: Do you check for the depth of anesthesia?

Yes, we check the depth of anesthesia by testing the mice´s response to a toe pinch. We added this information as a NOTE (line 174 of the revised manuscript).

13. 4.3, 4.4: what method of sacrificing is used in your experiment? How do you excise the footpad?

The sacrifice was performed using a CO_2 chamber following the Animal Care and Use Committees’ recommendations (step 4.3). The infected footpad was excised using sterile scissors and forceps (step 4.4). We added these information in the protocol section 4 of the revised manuscript.

14. 4.10: Do you plate this on the plates?

Yes, the limiting dilution is done in 96-well plates. We revised the manuscript to improve the description of this step (lines 239-241 of the revised manuscript).

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Steps to be filmed: 2 (2.3-2.5), 3 (3.1) and 4 (4.4-4.11).

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The figures adapted from a previous manuscript were published by PLoS Pathogens (https://doi.org/10.1371/journal.ppat.1007140), which applies the Creative Commons Attribution (CC BY) license policy. Under the CC BY license, authors retain ownership of the copyright for their article, but authors allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLoS journals, so long as the original authors and source are cited. No permission is required from the authors or the publishers, as cab be checked at the website http://journals.plos.org/plospathogens/s/licenses-and-copyright.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

As requested, the discussion section was extensively revised and improved to cover the referred items.

18. Please do not abbreviate the journal titles in the reference section.

As requested, the reference section was modified accordingly.

19. Please ensure that the table of the essential supplies, reagents, and equipment include the name, company, and catalog number of all relevant materials in separate columns.

As requested, we revised the Table of Materials and Reagents.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

This manuscript provide a detailed protocol on how to grow Leishmania in murine macrophage model. In my opinion this is the best model available. However, a step by step protocol with hint, pitfalls and practical advice is missing. This protocol will be useful to my research group as well as many of my colleagues will use this protocol. Our field of Leishmania research is missing a common protocol for in vivo analyses of parasite development. This manuscript and method is timely and provide a method that we all really need.

Major Concerns:
No comment

We thank are Reviewer #1 for the revision. We are really pleased that Reviewer #1 appreciates the importance and usefulness of this detailed protocol.

Reviewer #2:
So, infections of C57BL/6 mice with L. amazonensis as an experimental model of cutaneous leishmaniasis is a well-established model of cutaneous leishmaniasis. I wouldn't be surprised if many thousand of publications using this model have been published. There is no harm in revisiting established protocols and breathing new life into them. However, when updating old protocols, care should be taken to acknowledge those protocols and then include recent technical additions. The authors are presenting here a protocol for establishing L. amazonensis cutaneous leishmania infections in C57BL/6 mice. If done well, a publication like theirs could introduce new entrants into the field to well established methods.
That said there are several issues with the manuscript that should be addressed. Authors should revise the manuscript to reflect the fact that the protocols for experimental infections are already well established and that those protocols have led to numerous insightful studies on Leishmania pathogenesis. The authors should not suggest that the method that is presented is novel. Both the abstract and the Introduction should be revised accordingly to reflect the fact that this manuscript is not presenting a new protocol but rather a re-visitation of established protocols. The Current Protocols in Immunology originally published in 1991 has protocols for cutaneous Leishmania infections. A Reference such as that should be included in the revised manuscript.

We thank Reviewer #2 for the detailed revision and for acknowledging the importance of revisiting these established protocols that can assist in the introduction of new groups into the field.

We agree the protocol we describe in the present manuscript is a compilation of previous works and our goal is to revisit all these in a standardized detailed protocol. A step by step protocol with practical advices can be useful to several research groups that want to implement this method comparing their results with the ones obtained by other groups. So, as requested, we revised the abstract and introduction to reflect that we are not presenting a new protocol but revisiting well established protocols (lines 43, 47, 97 of the revised manuscript).

Additionally, we would like to emphasize that the Current Protocols in Immunology´s reference from 1998 was included as reference 22 and we added more citations of this specific reference, among other new references, along the manuscript.

They should also consider discussing the possibility of using ear infections as another cutaneous site for establishing cutaneous infections.

We agree with the reviewer and we included the intradermal ear infection as an alternative site for cutaneous infection (lines 435-439 of the revised manuscript).

In the protocols sect 1, they discuss media for cultivation of promastigote forms and the protocol and media for conversion of promastigotes to amastigotes. Although the parasites may change from the long-flagellated forms to the rounded forms, I am sure the authors are aware that rounded form are not necessarily amastigote forms. In this re-visitation of established protocols, it would be useful to use molecular indicators to confirm that a transformation has occurred successfully. For example, the abundant expression of gp63 in promastigote forms contrasts with limited if any expression in amastigotes. It is advised that the protocol includes molecular assays to confirm that transformation has been successful.

We agree it is important to acknowledge that not all rounded forms are necessarily amastigote forms. Previous studies standardized the axenic amastigote differentiation protocol with pH and temperature changes as we used here (Zilberstein et al. 1994 and 2019). Gene expression analyses of specific molecular markers can be performed to confirm the parasite transformation. Among these markers, amastin and gp63 that can be used to distinguish amastigote and promastigote forms as we highlighted in the revised manuscript (lines 463-469).

In the establishment on infections, they suggest to infect in a volume of 50ul. This is most likely much too much volume of liquid to inject in the hind foot of a mouse. 10ul may be more appropriate. Certainly, others have reported on this.

We agree smaller volumes could be easier to inject in the mice footpad, however not all labs have Hamilton glass syringes available for 10uL injections with appropriate accuracy. Besides, most guidelines for use of laboratory animals recommend the use of disposable syringes.

It is not clear when the weight of the infected tissue should be obtained. Should it be after dissection to free the tissue from bone or should it be after sectioning off the intact foot. In the former, the skill of the investigator in dislodging the tissue may influence the amount of tissue recovered, which will result in high parasite/weight values.

The weight of the lesion is calculated by subtracting the weight of the tube containing M199-pro-medium with the collected tissue from the weight of tube containing only M199-pro-medium, so after removing and discarding the bones (steps 4.5, 4.6). We added a NOTE in the revised manuscript recommending uniformity in the dissection step to avoid biased tissue recovery (line 224).

The limiting dilution method is a well-established method for estimating parasite burdens. Titus et al 1995, 1997 are two detailed publications that describe this method along with the statistical considerations. As presented in this manuscript, the method of analysis is flawed. The publications by Titus el al (1995) in Parasite Immunology on evaluation of the progress of Leishmania infections by limiting dilution assays are difficult to obtain. I assume that the difficulties with obtaining that publication may

explain in part why the authors of this manuscript didn't include that reference. This is unfortunate as they would have been made aware of the statistical considerations that need to be addressed when scoring limiting dilution assays.

We apologize for forgetting to include the original Titus' references in the first version of the manuscript. We have these publications and included the references for Titus 1985 and Titus 1997 in the revised manuscript (references 46 and 47).

Reviewer #3:

Manuscript Summary:

The manuscript "Murine in vivo infection with Leishmania amazonensis is a reliable method for a systemic evaluation of the host-parasite interaction" by Aoki et al, describes the protocol to obtain axenic amastigotes (or amastigotes-like) of Leishmania amazonensis, and describes how to perform C57BL/6 footpad infection, monitor the progression of the lesion, obtain amastigotes from the lesion and perform the limiting dilution. These protocols are extremely useful for those working with Leishmania parasites.

Major Concerns:

The main concern is the discussion, which should be improved. I think readers would like to be sure that the axenic amastigotes generated by this protocol are in fact amastigotes and no transitional forms. Do they meet the morphological criteria (non-motile, ovoid shape, and appropriate size), capacity of long-term propagation and infectivity in vitro and in vivo, molecular and biochemical differences when compared with cultured promastigotes?

We thank Reviewer #3 for the careful revision. We are pleased that the Reviewer #3 also appreciates the importance and usefulness of this detailed protocol for the *Leishmania* field.

The discussion section was extensively revised. We included information about gene expression analyses of specific molecular markers that can be performed to ensure the parasite differentiation. Among these markers, we highlighted amastin and gp63 (lines 463-469 of the revised manuscript).

Some Leishmania strains differentiate poorly into metacyclic promastigotes in vitro and the purification of metacyclic forms is expensive and time consuming. In these cases amastigotes harvested from infected tissue (or axenic amastigotes) may be used as inoculum. However, don't you think this is an unnatural way to initiate the infection in the mammalian host? I Think this should be discussed.

We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. Besides the reviewer observation that some *Leishmania* strains differentiate poorly into metacyclics, the use of axenic amastigotes also avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes

purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 460-463). So, as requested we revised the discussion section highlighting the most natural way to initiate the infection and the alternatives that can be used depending on the study purpose (lines 432-447).

Line 351- The sentence "Employing the protocol of axenic amastigotes differentiated from promastigote cultures can also be applied and produce comparable results." needs a reference.
We included references to support this sentence (line 460 of the revised manuscript).

Minor Concerns:
Line 112- I recommend using the term amastigote-like or axenic amastigote whenever the authors refer to amastigotes generated in vitro.

As requested, we revised the manuscript to refer to amastigotes generated in vitro as axenic amastigotes.

Line 117- The references Sinha et al, 2018 and Dumetz et al, 2017 are inadequate to justify the sentence "Use promastigote cultures that were passed in vitro less than 10 times". Can you provide a valid reference?

We included more references and modified the sentence to emphasize the virulence attenuation and genomic rearranges observed after *in vitro* passages (lines 135-136).

Line 119- It is important to provide references for "In vitro differentiation of L. amazonensis promastigotes into axenic amastigotes". For example: Flannery et al 2011 or your own nice references, Menezes et al, 2017; Mittra et al, 2017…

We included more references as requested (line 120 of the revised manuscript).

Line 125- The reference Zilberstein and Nitzan refers to the differentiation of L. donovani promastigotes into amastigotes. Does the same apply to L. amazonensis?

Yes, the same was observed for *L. amazonensis*. We included more references to support this (see lines 120 of the revised manuscript).

Line 131- What is the percentage of transformation from promastigotes into amastigotes with the proposed protocol? The sentence "NOTE: Count only non-flagellated parasites (amastigotes forms).", supposes that the transformation is not total, as already demonstrated by other authors. How do you suggest eliminating flagellated forms?

We modified the sentence to "count the non-flagellated parasites" since it is not required to eliminate the flagellated forms, which are significantly underrepresented after the axenic differentiation. In fact, it's rare to find flagellated parasites using this protocol.

Line 138- The parasites are injected subcutaneously or intradermally?

The parasites are injected in the subplantar tissue of the left hind footpad that is the most frequent site of inoculation in experimental *in vivo* infection studies. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose. We included more about this on the revised discussion section with more references (lines 434-439 of the revised manuscript).

Line 163- When obtaining amastigotes from mouse footpad lesions, it is recommended that the footpad surface be disinfected prior to excisement of the infected tissue to ovoid contamination.

We apologize for forgetting to include the disinfection step. The revised protocol includes ethanol 70% spray for disinfection (lines 215-219 of the revised manuscript).

Line 169- Another important NOTE: avoid placing the forceps and a scalpel directly into the medium as small volumes may be removed, resulting in an underestimate of tissue weight.

We appreciate this suggestion and we included a note about this (lines 230-231 of the revised manuscript).

Reviewer #4:
Major Concerns:
The authors have not referred to or entertained the possibility that axenic amastigotes may not be genetically or phenotypically similar to ex-vivo amastigotes. They are certainly not promastigotes but are the simply another in-vitro entity. Some explanation and substantiation in this regard is required.

Why would you want to initiate a Leishmania infection in-vivo with amastigotes? This has no physiological relevance. Leishmania infections are initiated by promastigotes, and more precisely metacyclic promastigotes.

We thank Reviewer #4 for the careful revision. We appreciate the reviewer concern and included more references to substantiate the protocol for the use of axenic amastigotes. The promastigotes used by most research groups are also axenic since they are not purified from sandflies. We agree that the use of axenic parasites may be not ideal, however the use of axenic amastigotes has been broadly accepted considering several issues on obtaining purified metacyclics. It is important to highlight that some *Leishmania* strains differentiate poorly into metacyclics. Besides this, the use of axenic amastigotes avoids the use of other methods that typically result in lower yields of infective parasites, such as: incubating promastigotes with specific but not-widely available antibodies for metacyclic promastigotes purification, or by density gradient dependent of metacyclics' LPG expression, as we discussed in the revised manuscript (lines 458-463). So, as requested we revised the discussion section to highlight the infection method alternative that better mimics the natural transmission of the parasite by using laboratory-infected sand flies to infect the animal´s ear dermis. However, this protocol presents some restrictions, such as the maintenance of sand fly colonies, which requires facilities not available for most research groups (lines 432-447).

High-dose infections have largely been abandoned in the field due to their questionable relevance to the early stages of physiological disease that is initiated with hundreds, not millions of parasites.

We also modified the introduction adding that the route, site or dose of inoculation influence the disease outcome (lines 84-85 of the revised manuscript). We discuss the sand fly usually inoculates a few hundreds of Leishmania metacyclic promastigote forms with the insect´s saliva (lines 433-434). Since, the goal of this manuscript is to revisit well-stablished protocols to assist in the introduction of new groups into the field, we keep the high-dose infection protocol that most groups are still using. Also, the lower doses are mostly used by groups working with sandfly transmission or saliva, an alternative not available for several groups.

Sub-cutaneous footpad inoculation is not a physiological site of infection, which is intra-dermal.
Sub-cutaneous footpad inoculation is associated with a non-physiological inflammatory response.
In the present manuscript, the site of inoculation is the subplantar tissue of the left hind footpad, which is reported as the most frequent site for experimental *in vivo* cutaneous infections. Intradermal injection into the ear is an alternative site of inoculation depending on the study purpose, however this protocol presents some limitations, as discussed in the revised version (lines 434-447).

The authors have not incorporated the role of dose or site of infection. The authors may not be aware of Cortes et. al. 2010 Mem Inst Oswaldo Cruz Vol.105(6) 736-745, showing dramatically different courses of infection following i.d. infection.

We agree that dose and site of infection are important factors to be considered We included the suggested reference (reference 27).

The authors have not mentioned the complications of immune cell recovery from the footpad, which is complicated by the bones, fat, and muscle associated with the footpad.

We included in the introduction section of the revised manuscript that for immunological studies, immunohistochemical assays from footpad tissue sections or even analysis of recovered immune cells from popliteal lymph nodes can be performed (lines 79-81).