Dear Review editor,
Dear Dr. Vineeta Bajaj,

Please find enclosed the revised version of our methods manuscript entitled: “Horizontal Hippocampal Brain Slices of the mouse brain.” that we recently submitted for peer-revision in the *Journal of Visualized Experiments.* We aim to contribute with this manuscript to the methods collection: “Preparation of acute hippocampal slices” by guest editor Dr. Felix Leroy.

Several editorial and reviewer comments were provided after the first revision of our work and we addressed all of the comments as detailed in the point-by-point response listed below. We believe that we could greatly improve the quality of our manuscript after carefully revising our prior submission.

We hope that you will find our revised manuscript suitable for publication in *JoVE.*

Yours sincerely,

Katharina Held

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We carefully proofread our manuscript and included several changes in the revised manuscript version.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 2.1.3: mention animal strain, age, sex.
2) 3.1: how do you ensure depth of anesthesia?

We adapted points 2.1.3 and 3.1 in order to provide some more detail. We checked all other protocol points and believe to have provided enough details to easily replicate the here presented technique.

• **Protocol Numbering:**
1) All steps should be lined up at the left margin with no indentations.
2) Please add a one-line space after each protocol step.

In the revised manuscript, all steps are lined up at the left margin without indentations with a space after each protocol step.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your

protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

A total of 2.5 pages was marked in yellow, excluding notes.

• **References:**
1) Please spell out journal names.

The reference list was adapted accordingly in the revised version of the manuscript.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MilliQ, Fine Science, Loctite 406,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have removed all commercial sounding language from the manuscript text. This information is now solely available in the Table of Materials and Reagents.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All here presented Figures were specifically recorded for the purpose to publish in this JoVE article. No material was published elsewhere before.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the protocol submitted by Van Hoeymissen et al. for consideration at JoVE titled "Horizontal Hippocampal Brain Slices of Mouse and Rat for secondary applications" the authors provide a step-by-step protocol for the production of horizontal brain slices from the rodent brain. This protocol is from a lab that has produced a number of key studies using this approach, including recent

examples, i.e. Held et al 2020. This protocol complements a number of previous protocols both in JoVE and elsewhere to describe the production of brain slices containing the hippocampus. I have a number of concerns that should be addressed by the authors to improve the understanding and usefulness of this protocol in relation to its intended audience:

Major Concerns:
1) I feel that the title of this protocol is somewhat misleading/confusing. I am unclear what a secondary application is in the production of brain slices. Presumably all experiments performed on brain slices as produced by the description here are the primary application? In the abstract, the authors identify a list of applications, with the first listed application being extracellular field recordings. This protocol contains representative results which describe extracellular field recordings in detail. This would not imply a "secondary application". Furthermore, the title mentions that this protocol is for mouse and rat brain slices. The provided protocol text only details murine techniques, and minimally discusses that this could be used in rats as well. Perhaps say this is primarily for mouse.

We thank the reviewer for his/her comments and adapted the title accordingly. We used the here provided protocol in the past in mice as well as rats. However, we agree with the referee's comment and will focus on one species.

2) I believe the authors make a bit of a faux pas in their summary: "apparent hippocampal slice protocols including a detailed systematic description are scarce", and introduction (line 117-118). There are indeed a great many protocols for describing slice preparation from mice and rats which provide a systematic approach both with recent advances - Booker et al., 2014 (JoVE); Ting et al., 2014 (JoVE); Hajos and Mody, 2009; Bischofberger et al., 2006, and with a traditional approach - Schwartzkroin, 1975, Newman et al., 1992. Several of these seminal studies are referenced by the authors, but a thorough review of these examples are absent.

The authors should carefully describe how their protocol differs from those previously produced for JoVE, or other protocol publications - especially in the context of horizontal slice preparation from the hippocampus - which have been extensively detailed in Booker et al., 2014. Without this clarification it does not signify a substantial advance of technique from these earlier studies.

We thank the reviewer for this comment. We did exclude the mentioned sentence from our Abstract and instead included some sentences highlighting the advantages of horizontal slice preparations over others (line 46 - 53). Furthermore, we included a new paragraph in the discussion of the revised manuscript, where we put our manuscript in perspective to other reported slice protocols (lines 569 - 581) and extended the paragraph in the discussion where we mention the advantages of horizontal brain slices in particular (lines 583 - 601).
However, we would also like to point out, that the protocol of Booker et al. 2014 that was mentioned by the reviewer is not providing a detailed description of the horizontal slicing process itself, but rather focuses on the patch clamp recording of parvalbumin-expressing interneurons. The details of each individual slice step and used materials are lacking in our opinion in the protocol as well as in the supporting video. Further, Booker at al., employ a different cutting angle for their preparations which was reported to be of particular importance for proper mossy fiber preservation (Bischofsberger et al., 2006).

3) The text contains a number of grammatical inaccuracies and unusual sentence structures which should be addressed. A careful proofread of the manuscript text would greatly benefit the protocol. A number have been highlighted in the minor comments section.

We thank the reviewer for his/her attentive reading and have made several adaptations within the manuscript in order to improve the language.

4) It is unclear to me why the authors discuss the pros and cons of organotypic slice cultures in the introduction. It is somewhat distracting and unrelated to the protocol presented. This should be toned down or removed entirely.

We can agree with the reviewer's comment. In the revised version of the manuscript we excluded organotypic cultures from the introduction.

5) I have a concern about the storage and preparation of ACSF solutions, from a number of the protocols referenced (Ting et al., 2014; Bischofberger et al., 2006) and those not mentioned (Booker et al., 2014), these studies, as well as a great many other published physiological reports suggest using solutions of osmolarity 290-310 mOsm (within the physiological range). The authors state that they use a slicing solution with osmolarity of 205-215 mOsm (which is entirely non-physiological) - however the ACSF recipe they provide has a theoretical range of ~300 mOsm. Can the authors please comment?

We thank the reviewer for noticing that rather dramatic mistake. These were typo's in our previous manuscript version. We adapted the osmolarity to an isotonic solution ~300 mOsm.

Furthermore, the statement that 1x physiological solutions can be kept for up to 1 week is rather at odds with the majority of labs, which make their 1x solutions on the day of slicing and only keep for 1 day, perhaps 2 days under exceptional circumstances - see method details outlined in Bischofberger et al., 2006.

We do agree with the comment of the reviewer concerning the storage of the ACSF solution. In fact, we standardly prepare our ACSF solution at the day of experimentation in order to guarantee high quality slices. Only in very rare cases, we do prepare it the evening before. We know from peers that performed recordings without any issues when storing the ACSF at 4°C for up to one week and similar suggestions are even published in other JoVE brain slice protocols (Ting et al., 2018). However, as we intend to provide a protocol that guarantees consistent high-quality slice preparations, we adapted this part in our manuscript as suggested by the reviewer and as it is standardly performed in our lab (Step 1.2.1).

6) The authors discuss how "Only a tight control of all these key factors is allowing the preparation of continuous high-quality brain slices, ultimately rendering stable and comparable results". It is not clear how the authors control for these factors in their protocol either. Either tone this down or include quantification or qualification of quality controls, beyond only a fEPSP with a large fibre volley.

We did provide more detailed information concerning some of the key factors mentioned in the sentence before (lines 98 - 101). These details are now included in the relevant steps of the protocol and should suffice as a guideline for setting up the experiments. Furthermore, we mention other quality controls in our revised manuscript version such as the addition of high potassium in calcium-microfluorimetric experiments (step 7.8), microscopic cell assessment or a DAPI nucleus staining (lines 629 - 631).

7) It is unclear what the methods are for fEPSP recording or calcium imaging, as depicted in the representative results. I appreciate that this protocol focuses on slice preparation, but much of the

method detail is limited to the results text itself. I would suggest adding a short section to the methods describing one or both/either protocol.

We included two short sections with the necessary steps for fEPSP and calcium recordings (steps 6 and 7) and referred to other manuscripts providing detailed protocols (lines 398 - 399).

The authors then provide qualitative detail about what constitutes a good slice or a "bad" slice. However, the examples given and detailed could equally arise due to errors in slice handling in terms of the fEPSP recording, solution preparation, or other errors from the specific experiments outlined, and not simple due to slice quality. None of the measures used rule out these other factors to poor recordings, as such they do not confirm slice health. These other sources of error need to be specifically addressed or an independent confirmation of slice health i.e. condensed nuclei from DAPI labelling or a representative chart showing a reduced input-Output curve from an unhealthy slice. The discussion (Line 424 onwards) should also be modified to reflect this.

The reviewer is correct that other technical or experimental problems could also have an impact on the quality of the measurements and could be interpreted as bad slice quality. However, for electrophysiological recordings Input-Output curves and stable baseline recordings are the best measure to judge the slice quality. Moreover, a high-quality slice might appear unhealthy due to these other issues, but a low-quality slice will never look like a 'healthy' one. Therefore, in our opinion, these measures still present a valid quality control during electrophysiological experiments. Nevertheless, as requested by the reviewer, we did discuss this pitfall in the revised manuscript (lines 626 - 631) and also proposed to build in a quality check in form of a DAPI staining. Additionally, Input-Output curves for 4 representative low-quality slices are included in Figure 3D-F.

Regarding the presented input-output curve, it would be more appropriate to show both the input-output curves for both the afferent fibre-volley and then the fEPSP plotted against fibre-volley, which better reflects the amplitude of fEPSPs produced in response to the intra-slice control.

We agree with the comment of the reviewer and have adapted the Input-Output curves in the revised version of the manuscript (Figure 3D-F).

8) Given that the authors show LTD in the absence of Bicuculline or NMDA, but LTP in the presence of either drug, can the authors comment in the results and/or discussion that the protocol they are employing is not an LTP induction protocol per se, but rather a mechanism to induce heterosynaptic activation of synapses, which can lead to bi-directional plasticity. Again, given the absence of any method detail, this panel is confusing and at odds with the LTP narrative being put forward.

Independent of a hetero- or homosynaptic mechanism, the here employed protocol (4 HFS of 100Hz with 5 min ISI in presence of Bicuculline) is clearly inducing LTP as it results in a long-lasting increase in the fEPSP signal (up to 60 min after conditioning), which is the definition of Long Term Potentiation. Also note, that this long-lasting increase is in the absence of Bicuculline and NMDA itself, as the drugs are only applied during the induction protocol and omitted directly after the last HFS pulse. It is commonly known that LTP induction in the MPP is requiring a stronger induction protocol than in other regions of the hippocampus (e.g. CA1) and the use of Bicuculline is therefore often recommended in order to facilitate the LTP induction. For terms of simplicity, we did remove the red time course in Figure 3H and only focus on the LTP examples itself now. The exact induction protocol is explained in more detail in the Figure legend of the revised manuscript.

9) If detailed protocol methods are not provided, remove Calcium Fluorimetry representative results and discussion. As many JoVE videos have been produced on calcium-imaging techniques, going into

more detail than presented here (i.e. Ma et al., 2011; Laclar et al., 2012; Irwin and Allen 2013; Leinweber et al., 2014, amongst other protocols published elsewhere) it is unclear how useful further methodology would be, trying to cover many aspects of their usefulness.

We have included a more detailed description of the calcium microfluorimetric experiments in the protocol section (step 7) of the revised manuscript and referred to relevant JoVE videos that provide further details (lines 398 - 399).

Minor Concerns:
Line 29: "Highly preserve the integrity" -subjective, remove "highly"
Line 30: "Paths" should not be pluralised in "Perforant Paths"
Line 36: "The hippocampus includes a high degree" - includes should be replaced with "receives"
Line 39: "Brain slices are the number one ex vivo choice when exploring neurophysiological functions of the hippocampus" - that slice preparations are the "number one…choice" is highly arguable and dependent on the experimental aims/method.
Line 64: This sentence has a very confusing syntax and tense - "A dense network of informational flows in the form of fiber pathways is accomplishing a tight hippocampal connection to internal and external brain structures." Consider revising.
Line 69: "neatly" - subjective and implies a level of design. Consider replacing with an alternative such as "highly conserved laminar organisation"
Line 84: "secure" should be replaced with "preserve"
Line 137: MilliQ water - MilliQ water comes in many grades. Is it possible for the authors to comment on the quality of water - Grade 1, 2, or 3. Ultrapure double deionised or just single distilled.
Line 151-152: From experience ACSF does not emerge from the freezer as a semi-slushy solution, does this require shaking/homogenisation? Please elaborate
Line 183: What makes this an ultra-freezer? Is it different in some way from a standard -80°C freezer?
Line 189: Point 2.2.4 - does this specifically have to be a 35 mm culture dish? Could it be any small beaker/container on ice? A 35 mm dish maybe not appropriate for a larger brain - i.e. a 6 week old rat, which this protocol states it would be suitable for.
Line 196: Point 3.2 unusually structured and the meaning is unclear
Line 214: Please clarify why is meant by diffusional forces - I am not sure this strictly applies to this procedure. A more simple terminology might be better.
Line 238: The use of the word "further downwards" is somewhat unusual given the previous use of anatomical directions. Indeed, the "downward" direction is actually in the dorsal direction, if I have understood that the brain slicing method is very similar to Bischofberger et al., (2006) or Booker et al., (2014). Indeed, the vibratome used (Leica VT1200S) actually brings the brain to the blade, as such terminology is not correct in either case. Perhaps consider replacing with "more dorsally in the brain", for example.

We thank the reviewer for pointing out these errors. We did revise all of the upper comments in our revised manuscript version.

Line 417: "hippocampal input pathways and DG-related processes such as epilepsy", presumably the authors mean experimental epilepsy? Please revise.

We removed the example of epilepsy from the sentence.

Reviewer #2:
Manuscript Summary:

The manuscript describes a clear protocol on how to produce healthy brain slices for physiological recordings. Some of the critical steps, which remain under the radar in regular material & method sections of papers, are very well described. The authors show some of the applications that can be performed on hippocampal slices and I appreciate that it is indicated clearly how healthy slices can be distinguished from bad slices.

One major comment is that is not clear/obvious what the advantage is of the described horizontal hippocampal slices above sagital slices in full brains or even 'chopped isolated hipocampi'. This should be better explained.

We thank the reviewer for this very relevant comment. We extended our manuscript by adding some more detailed explanation of the advantages of horizontal brain slices over other slicing techniques to the Abstract (lines 46 - 53) as well as the Discussion (lines 569 - 601) sections.

Minor Concerns:
Title:
the description "for secondary application" is very unclear and doesn't add anything to the message. This part can be deleted or should be explained more specific. What is a primary application by the way?

We thank the reviewer for his/her comment and adapted the title accordingly.

Abstract:
Some illogical sentences are present, probably due to size restriction:
"electrophysiological recordings, such as field potentials, whole-cell patch clamp and calcium fluorometry" --> calcium fluorometry is not an electrophysiological technique. In addition calcium fluorometry is not the only light microscopic technique that is used in hippocampal slices: e.g. potential measurements, measurements of pH, other ions synaptic release,… not to forget optogenetic techniques. --> I suggest to rephrase to electrophysiological recordings, light microscopic measurements as well as molecular biological and histochemical techniques.
Although the manuscript describes horizontal brain slices, no notice of the advantages/difference of sagittal versus horizontal brain slices is even mentioned. In general, the abstract should focus more on the need for, and advantages of horizontal brain slices, rather than discussing the use of brain slices in general, as these are mostly referring to sagittal brain slices. Advantages compared to using isolated hippocampus cut with a tissue chopper should also be highlighted.

We adapted the Abstract accordingly in lines 42 and lines 46 - 53.

Line 75: Brain slice protocols are laborious, time-consuming and often result in the loss of connections…. --> it is mostly not the brain slicing that is laborious, but the subsequent experiment and analysis that is performed on the cells of the slices. Any primary cell culture would take more time than brain slicing. Only the loss of connections is an issue in my opinion.

The reviewer is absolutely right with this comment, so we deleted this part in our revised manuscript version.

102: "organotypic hippocampal slices, acute hippocampal slices are usually prepared from adult rodent brains" I agree that organotypic brain slices are usually prepared from ebryos, but it is not true that acute slices are mostly from adult. The age of the mice to prepare acute brain slices is dependent on the research question (developmental versus adult). Rephrase or delete the sentence.

In our revised manuscript, we deleted the complete section discussing organotypic slices as was suggested by another reviewer and with it also the statement addressed by the reviewer in this comment.

112: optimal slice quality is highly dependent on ideal experimental conditions, including the age of the animal, the method of euthanasia, the speed of dissection and slicing, the slicing solutions and parameters (e.g. slicing speed) as well as the conditions for slice recovery --> Although not specifically mentioned as a topic of this work, trans-cardiac perfusion of the animal with ice cold PBS before the dissection, is in some cases essential (especially for old rats) to produce high quality brain slices. This might be mentioned somewhere is the manuscript.

We thank the referee for this comment and have included this additional information in the revised manuscript (lines 614 - 615).

166: If older animals are used, slice and recovery solutions may have to be adapted accordingly4 (e.g. NMDG+-based solutions) in order to preserve the brain health of the acute slices. --> in the same line of the previous comment, it might be worthwhile to add a reference for perfusing animals and for adding additional Mg2+ to block NMDA receptors and excitotoxicity).

We added a paragraph in the discussion where we mention several measures that could be taken for slice health preservation. Appropriate references are provided in the same paragraph (lines 603 - 615).

3.11 Use a filter paper strap to pick up one hemisphere with diffusional forces, thereby not damaging the tissue. --> it is not clear to me how this step prepares for the next steps, because, if I didn't' miss it, it is not written here which side should be facing the bottom (=glue) on the specimen plate. In fact, this only becomes clear in point 4.1.

We thank the reviewer for this comment. We revised this part in step 3.1.1 and 3.1.2 in order to make the orientation better comprehensible.

The left part of figure 2D shows this orientation but the right part of Fig 2D is a top view of the positioning, which does not fit the orientation of the left part of the figure. Use a separate panel number for both figures or any other way to make this more clear at first sight.
In addition, please mention why the dorsal side should be 'dried' in the next step (probably to glue it on the specimen plate?).

We adapted the Figure 2 and Figure 2 legend accordingly.

230 Important: the entire dissection procedure should be performed as fast as possible ◊ indeed a critical step, so please indicate a maximal time window in which the brains should be dissected and submersion in the slush. (Max 5 minutes, but can be faster?)

We added a time frame to the protocol in order to guarantee slice quality (lines 258 - 260).

Results:
Indicate the pipette resistance used for field potential measurements.

The pipette resistance of ~ 2 MΩ was included in the revised version of the manuscript (step 6.1 and line 416).

e.g. line 276: Is a solution heater used in line with the gravity controlled perfusion system? Or another heating system? Is this not essential for normal synaptic facilitation?

We thank the reviewer for this critical question. We did not use a solution heater in line with the gravity-controlled system, as it is practically very challenging to accomplish a **constant heating** of all tubes of a **multi-barrel system**. However, this system allows us to perfuse several different drugs during one slice recording. Although we agree that it would be more physiological to heat the slices during the recordings, it is clearly not essential, as we are able to obtain LTP in several hippocampal fiber pathways even at RT. The temperature might be seen as a limitation of our specific recordings that are represented here, but as this protocol is focusing on describing the brain slice procedure rather than the implementations thereafter, and simply shows the fEPSP recordings as an example result, we don't consider this comment as relevant to address for the here submitted manuscript.

Figure 3: terms 'positive and negative' examples are weird. Is 'example of a good quality slice' and 'example of a bad quality slice' not better?

We did adapt the Figure legend accordingly.

Reviewer #3:
Manuscript Summary:
This paper describes a protocol for obtaining rodent hippocampal brain slices that preserve the integrity of the perforant path - hippocampus and mossy fibers - hippocampal CA3 circuitry for use in electrophysiology and fluorometry experiments (and other suggested secondary applications). The protocol is written in a clear and objective language and described in sufficient detail for an independent user to follow it and obtain good quality slices for the described applications. There are however some details that need reviewing/clarification and are listed below.

Concerns:
1) First of all, although the manuscript is written in a clear/objective language, the use of English and phrasing needs reviewing in several instances as there is a continuous mixing of verb tenses (past, present, future, infinitive) throughout. For example, in lines 63-65. "A dense network of informational flows in the form of fiber pathways is accomplishing a tight hippocampal connection to internal and external brain structures" (should read, for example, "(…) which accomplishes"). Another example, in lines 82-84: "Second, the circumvention of the blood-brain-barrier (BBB) and the wash-out of endogenously released molecules before the start of the experiment are making it possible to study the effect of compounds and drugs with relatively precise dosage control." (should read, for example "make it possible"). These are just two examples from the introduction, but several other similar issues occur throughout the manuscript, especially in the introduction and discussion. I would advise the authors to review the use of English thoroughly.

We thank the reviewer for this constructive criticism. We carefully reviewed the use of English grammar in our manuscript.

2) Regarding the introduction, the paragraph on the comparison between brain slices and organotypic cultures seems unnecessary and not pertaining to the objective of the paper. I agree that some of advantages listed on that paragraph are important advantages of brain slices, but could be included on the second paragraph of the introduction, where other advantages of brain slices are listed/discussed.

We do agree with this reviewer's comment and adapted the Introduction accordingly. The paragraph of organotypic slice cultures was erased and general advantages of brain slices were

integrated in the paragraph before (lines 76 - 96).

3) On line 105/106, it reads "which results in higher amplitude signals obtained by extracellular recordings, thereby decreasing the signal to noise ratio". It should be "increasing the signal to noise ratio".

We thank the reviewer for his/her attentive reading of the manuscript. We adapted this statement (now in line 619).

Regarding the protocol itself:
4) On 1.1.2 (line 140) and 1.2.2 (line148) the target osmolarity of the solutions are described as 205-215 and 220-230 mOsm respectively. However, osmolarity for brain slices' solutions is usually described at 300 mOsm and solutions are typically expected to be in the range of 300 +/-10 mOsm. Can you please double-check your osmolarity values?

We thank the reviewer for noticing that rather dramatic mistake. This was a typo in our previous manuscript version. We adapted the osmolarities to 305-325 mOsm.

5) On 1.2.1 (line 146), the instruction starts with "warm up (…) to RT". This phrasing seems to imply that the solution should be actively warmed up. Could the sentence be rephrased? For example "Remove from 4º C and allow solution to reach RT".

This comment does not apply anymore to our revised manuscript version, as we changed the timeline of the preparation. We now propose to prepare ACSF fresh at the day of experimentation (step 1.2.1).

6) Also on 1.2.1 (line 146) it is stated that the pH of the carbogenated solution should be 7.3 to 7.4. Does this mean that the pH solution is not measured after preparation and before carbogenation? Considering that the pH drops approx. 0.1 in the carbogenated solution, it is usual to measure the pH before carbogenation, considering the pH drop (i.e. setting the pH before carbogenation to 7.4-7.5). This facilitates adjusting the pH to the correct range, when necessary.

We do agree that an extra pH check directly after solution preparation can be performed in order to notice potential mistakes that occurred during the preparation of the solution. However, it seems to us that the pH check is of actual relevance before the start of the experiments in order to guarantee the proper conditions. By thoroughly following the here described protocol the pH should be in a good range before the carbogenation. Therefore, a prior pH check can be useful, but is not necessarily required. We added the expected pH before carbogenation to steps 1.2.1 and 1.2.3 to provide the reader with this extra information.

7) In line with previous comment, the protocol should also describe how to proceed to adjust the pH when it is not at the target value.

We inserted a note in steps 1.2.2 and 1.2.4, where potentially necessary pH adjustments are discussed.

8) Still regarding the pH, the protocol does not describe what is the target pH level of the cutting solution (and if it is measured at all) - this pH should also be checked and adjusted.

We thank the reviewer for his/her attentive reading. Indeed, we missed to report the pH check for the slicing solution. We included this in step 1.2.4.

9) Regarding tables 1 and 3, pertaining to protocol steps 1.1.1 and 1.2.2, the authors should review the listed concentrations. In table one, since it's a 10x solution, shouldn't the concentration be 10x higher than listed (for example, KCl 25 mM instead of KCL 2.5mM), since it will be diluted in aprox. 1:10 in step 1.2.2, for a final concentration of 2.5 mM?

We thank the reviewer for that comment. We adapted the concentrations in Table 1 accordingly in order to facilitate the protocol.

10) Still regarding tables 1 and 3, to facilitate reproducibility, the exact molecular weight of the reagents used should be listed (as they can vary according to each manufacturers' specific formulation). The weighed mass of each reagent for each of the solutions should also be listed.

The exact molecular weight of the products used and the weighed amounts were added to Table 1-3.

11) On 1.2.2 (line 148), the use of a plastic beaker is suggested. Glass labware should always be preferred to plastic labware whenever possible.

The reviewer is right with his/her comment. Therefore, we erased the plastic out of the revised manuscript (step 1.2.3). We do usually use a plastic beaker because of the storage in the -80 freezer which bares the risk to break the glass. As this is not a crucial point of the protocol, we leave it open for the experimenter to decide which beaker to use.

12) On 3.6 (line 203) the use of a fine brush to clean the extracted brain is recommended. I would advise against this method of cleaning as it can cause inadvertent damage. The brain can be cleaned by gently rinsing it with aCSF poured by a pipette.

We thank the reviewer for this helpful tip. The manuscript was adapted accordingly.

13) On 3.9 (line 209), the instruction is confusing. I understand that it is hard to explain the cut through words, but could the authors try to make this instruction clearer?

We tried to express the orientation of the cut in more clear words in step 3.9.

14) Regarding the recovery/holding chamber (step 5), the chamber is listed on the table of materials as "self-made" and the materials need to build one are listed on the legend of Figure 2. However, can you provide a reference for a commercial alternative? (for example, the one listed on the manuscript's reference 23).

In the revised manuscript version, we do point out the commercial availability of recovery chambers (step 5.1), but do not name any because of the JoVE journal restrictions that don’t allow to use commercial language.

15) Regarding the discussion: I missed some words on the comparison of this protocol for the preparation of brain slices with other brain slices' protocols currently on JOVE. Although novelty is not an objective of a protocol/methods paper, it should be discussed in comparison with other available methods (especially on the same journal/platform).

We included a short paragraph in the discussion of the revised manuscript, where we put our manuscript in perspective to other reported slice protocols (lines 569 - 581).

Reviewer #4:

Manuscript Summary:

The manuscript by Van Hoeymissen et al. describes a method to obtain horizontal slices of live, healthy murine hippocampus that preserves performant path connections for use in electrophysiology, calcium imaging or any number of secondary experiments. The authors' goal is to present the protocol clearly and in detail to ensure consistent production of healthy slices. The authors give examples of electrophysiological markers to assess slice health and present examples of calcium imaging and electrophysiology experiments. This is a well written manuscript, but has several shortcomings that limit its usefulness over other protocols.

Major Concerns:

1. There is no discussion of the reasons why the proposed protocol results in healthy slices compared to other protocols, nor why horizontal slices are necessary to obtain perforant path synapses.

We never intended to claim that our protocol is superior in providing healthy brain slices in comparison to other protocols. We simply want to communicate a detailed slice protocol that yields a high success rate in our hands. The major advantage that we see in using that protocol is indeed the preservation of the perforant path next to all other hippocampal pathways, which creates the opportunity to study multiple circuits by use of a single slicing technique and even allows multiple-circuit studies within one slice. We explain the advantages and differences to other slice protocols in some more detail in our revised manuscript (lines 569 - 601).

2. No data is provided for quantifying the purported success rates of obtaining healthy slices; therefore, it is impossible to determine if this method is consistent and should be followed. This should also identify which steps are absolutely critical to the health of the slice.

We thank the reviewer for his/her comment. We inserted a new paragraph (lines 603 - 615) discussing the critical steps for healthy slice preparations and state the success rate obtained in our lab. Further, we also point out that other measures might be necessary in order to obtain high quality slices in different test conditions.

3. One of the stated goals is give precise details that are lacking in other descriptions of slicing so that there is no question on how to do this. However, the authors remain vague at critical points. For example, at the end of the dissection and slicing description on lines 230 and 250 it is noted that it is important to work fast. This is one of the most critical aspects of preparing healthy slices! The authors should be more explicit. At what points do you need to be fast? How fast? Right from the start, the mouse needs to be decapitated and brain removed and placed in the sucrose-acsf slush within 30-45 seconds.

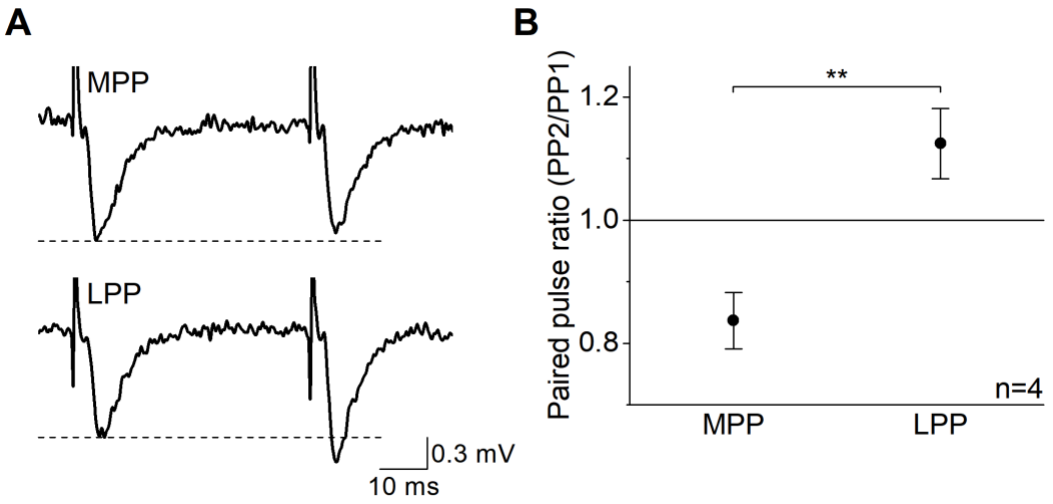
We thank the reviewer for noticing this inaccuracy. We added a time frame to the protocol in order to guarantee slice health (lines 256 - 260).

4. Experimental example: The authors example of an input-output curve isn't the best, stimulation intensities start high (almost at the 50% intensity) and the error bars suggest wide variability between slices. One should be provided that has a wider span of intensities and fit with a Boltzmann equation and calculate V50, not dose-response function.

We do agree with the reviewer's comment and provide better quality Input-Output curves in the revised manuscript (Figures 3D-F), now showing a wider range of stimulation intensities. We removed the dose-response function fit.

How do the authors know they are recording from MPP? To assess the health of the slice/synapse the authors also need to test paired pulse ratio. The MPP to DG synapse should have paired pulse depression whereas the LPP to DG synapse should exhibit facilitation.

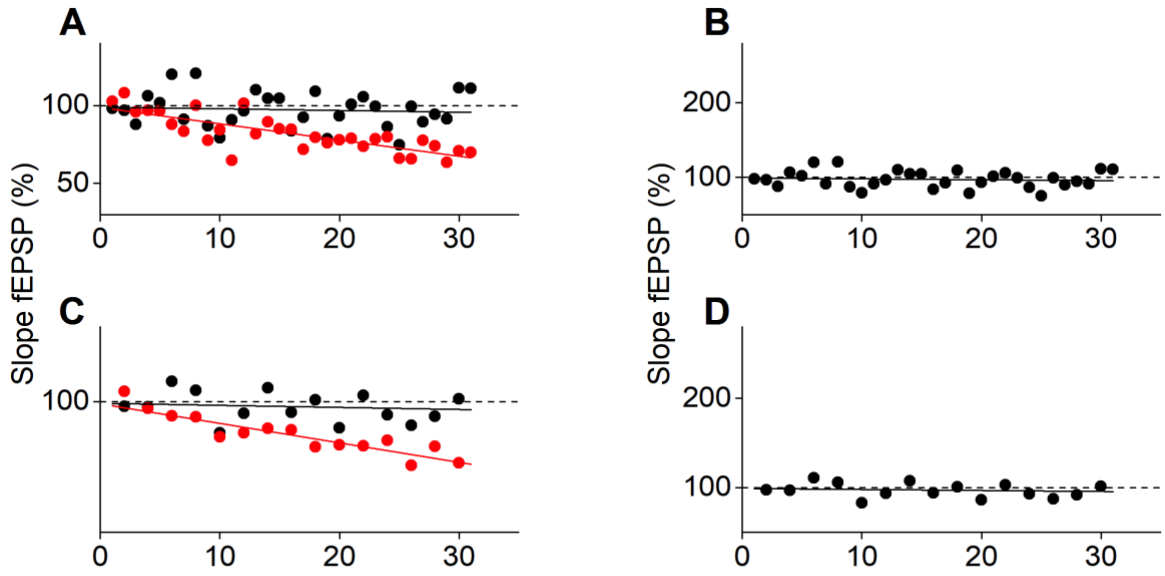
We do not fully agree with the reviewer statement that a paired-pulse protocol would give us any additional information concerning the **slice health**. The reviewer is absolutely right that a paired-pulse protocol can be useful in order to verify the proper electrode positioning, differentiating the MPP from the LPP. Indeed, we occasionally do use this protocol in our recordings in order to verify the right pathway. See below the results from paired-pulse recordings in MPP and LPP within the same slice: A) Paired-pulse traces (with 50 ms ISI) recorded in the MPP and LPP of four individual slices, B) Statistics of the paired-pulse ratios showing PPD for recordings in the MPP and PPF for recordings in the LPP. We could monitor PPD in 100% of cases when recording in the MPP (a total of n=10). However, it should be also noted that several manuscripts report already that PPD is not always restricted to MPP and PPF not to LPP and even more useful test protocols were brought forward (Petersen et al., Neuroscience, 2013). Therefore, the best way to guarantee the investigation of the right perforant path is the careful positioning of small diameter electrodes. As the scope of this manuscript was not to provide a protocol for MPP recordings but solely focuses on the brain slice preparation procedure itself, we do prefer to not show and discuss any such recordings in the here presented manuscript. In order to anyway address the reviewer's comment, we did insert a few sentences pointing out the importance of a proper control for MPP recordings (lines 422 -426).



Also, be precise on the definitions of a healthy slice: How small is too small for the fEPSP to fiber volley ratio? How small is too small for the fEPSP slope/amplitude? How much variation in the fEPSP is acceptable/unacceptable? The example in 3E is quite variable.

We thank the reviewer for this notification. We inserted some specifications in the results part (lines 466 - 469). This also includes a cut-off baseline alteration value that will hopefully clarify why we consider the presented baseline to be stable. We do agree with the reviewer that the baseline example in Figure 3E shows some variations. Variations are in our hands not untypical and may be caused for example by perfusion level differences. Nevertheless, we consider the recording shown in 3E stable, because the overall change of the baseline is below 5% and therefore in an acceptable

range. The variations may also just seem more pronounced because of the representation of a single example recording which yields more variations than seen when the mean of several recordings is presented. Furthermore, the scale of the graphs might make the data look more variable. Find below a Figure showing the same data as presented in the manuscript (Panel A) compared to the same data presented on a broader Y-axis range (Panel B) that would be typically applied for LTP recordings. Furthermore, Panels C and D show the same recording represented with mean fEPSP values of every 2 min instead of every 1 min. The Y-axes are similar as seen in Panels A and B above. These representations show an acceptable stability of the baseline recording.



Minor Concerns:

1. The sentence starting on line 63 and ending on line 65 does not make sense.
2. The sentence starting on line 103 indicates that several reasons will be presented to illustrate the importance of hippocampal slices. The following sentences only provide two reasons. Two is not several.
3. Protocol, line 141 and 150. The osmolarities are quite low. What type of osmometer should be used?
4. Protocol, The protocol lacks a description to prevent calcium phosphate from precipitating out of solution.
5. Protocol, line 137. The 10x presolution does not appear to be 10x.
6. line 196. some IACUCs require a small animal guillotine rather than scissors to decapitate.
7. The description of the dissection and orientation of the brain and Figure 2c-f is not clear. It almost looks like these are saggital sections. Why do the authors not block the brain, cutting off the prefrontal cortex and cerebellum? Are the hemisopheres glued dorsal side down or ventral side down?

All minor comments above were addressed accordingly in the revised manuscript version.

8. Describe why stimulation intensity is set at 50%.

This explanation is provided in the Results section (lines 444 - 447).

9. Describe why it is difficult to induce LTP at the MPP synapse.

We inserted a short explanation why LTP induction in the MPP is difficult to achieve (lines 458 - 461).