**Changes recommended by the JoVE Scientific Review Editor**

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

We have carefully read the manuscript and we have used the spell-check feature of our word processor software. The manuscript should now be free of errors/typos.

1. **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 you answer the “how” question, i.e., how is the step performed?**

We have revised the protocol and we have merged some steps according to these indications. Some notes were included within the step itself in light of their relevance to the movie.

1. **Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action.**

Thanks for specifying this aspect. Section **8 – Brain slice preparation and maintenance** contains reference to previous work on how to obtain the *partially disconnected brain slice* that we use in this protocol. Other than that, the procedures related to the preparation of brain slices are standard and can be found throughout the literature. Thus, this protocol section is not highlighted for filming. However, while reminding the reader to the appropriate reference, we have included the description of these steps in section 8 for a matter of completeness, as we feel that it may add useful information.

1. **There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:**

**1) Can “mapMEA.m” be provided to readers as a supplementary code file?  
2) 5.7: reference Table 2 here for ACSF composition.**

We have improved the protocol’s description by adding some important details that we had overlooked, while removing some others that may have generated confusion.

The script *mapMEA* is available upon request. Thanks for pointing at this fundamental detail. We have added a statement in the Introduction. The contact details to request the software are reported in the Acknowledgment section.

We have added the reference to Table 2 in the first note to section **2 –** **Preparation of stock solutions**, so that the reader may rapidly check both stock and final solutions composition.

1. **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. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.**

We have revised the protocol highlight (highlighted in YELLOW in the revised version of the manuscript) as per the above specifications. The revised version contains some steps that were not initially included, whereas procedures that will not be filmed have been completely removed. The total length conforms to the guidelines.

* **Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.**

We have merged sentences where appropriate throughout the protocol. We hope that it now reads smoother and that it responds to the journal’s standard.

* **The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.**

We have read the highlighted protocol as if were narrating the movie. It now reads smoothly and it forms a cohesive story on how to go through the steps in order to achieve the final experimental output.

* **Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.**

We have rearranged the text in order to highlight complete sentences.

* **Notes cannot be filmed and should be excluded from highlighting.**

Thanks for stressing this aspect. We have included within the steps themselves those notes that are relevant to the movie.

* **Please bear in mind that software steps without a graphical user interface/calculations cannot be filmed.**

We provide a step-by-step tutorial on how to use the software for the electrode mapping (*mapMEA*). The software uses a graphical user interface (GUI) that will also be shown in the movie.

1. **Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.**

Thanks for stressing this. We have indeed realized that the Discussion section was too tilted towards scientific rather than methodological aspects. We have therefore re-written the Discussion entirely following the above suggested sub-headings.

1. **References: Please move the in-text http web links into the reference list, and use superscripted citations. (e.g. Line 103, 582).**

The web links were removed from the text and added to the reference list. Please, note that since we have discovered that a website has been taken down, we have changed the reference to point to the original publication by which the website was inspired.

1. **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 Multichannel Systems, MATLAB (The Mathworks), etc. 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.**

Thanks for reminding this. We have carefully checked the text and there should be no mention of commercial identifiers.

1. **Table of Materials: Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as elastomeric sealant, enzymatic cleaner, etc.**

We have carefully revised the table of materials and we have actually found that some materials or descriptions were missing. Thanks for pointing at this. The Materials Table should now be exhaustive.

1. **Please define all abbreviations at first use.**

We have screened the text deeply and all the abbreviations should now be preceded by the full-spell.

1. **Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.**

We have checked the text and everything seems correct.

1. **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 our figures are original.

**Reviewer #1**

**Manuscript Summary:**

The ms "Recording and Modulation of Epileptiform Activity in Rodent Brain Slices Coupled to Micro Electrode Arrays" by Gabriella Panuccio, Ilaria Colombi, Michela Chiappalone describes a protocol to perform MEA recording and modulation of 4AP-induced epileptiform activity in rodent brain slices. It illustrates how to prepare customized MEA chambers, to cut slices and to perform the recording using a customized MATLAB script, and they show ictal activity modulation through electrical stimulation at 1Hz.

**MAJOR CONCERNS:**

1. **The protocol is well described into details and all the steps are clearly explained. Recording epileptic activities in brain slices in MEAs is not new and has, for example, already been published in JoVE.**

We thank the reviewer for raising this point. A deep search of the literature yielded reports on spontaneous discharges and pharmacological studies (some of which are referred to in our manuscript), but we could not find any paper describing electrical modulation of limbic ictogenesis using MEA and the rodent brain slice preparation used in our work.

We have also searched JoVE archives specifically and we could find the following two papers, which, however, differ substantially from the experimental method described in our work:

* The paper *Direct-current Stimulation and Multi-electrode Array Recording of Seizure-like Activity in Mice Brain Slice Preparation* by Lu et al. (J. Vis. Exp. (112), e53709, 2016, doi:10.3791/53709) describes recording and modulation of epileptiform activity in mouse brain slices using MEAs and aims at evaluating direct current stimulation parameters for modulating the thalamo-cingulate pathway. In our work, we describe a different brain slice preparation (combined hippocampus-cortex slice) and we build on previous literature (Barbarosie, M. & Avoli, M. *CA3-driven hippocampal-entorhinal loop controls rather than sustains in vitro limbic seizures*. *J Neurosci.* 17 (23), 9308-9314, 1997) to demonstrate that it is possible to replicate modulation of limbic ictogenesis by means of electrical stimulation delivered directly through MEA electrodes in the subiculum.
* The paper *Multi-electrode Array Recordings of Human Epileptic Postoperative Cortical Tissue.* J. Vis. Exp. (92), e51870, 2014, doi:10.3791/51870) by Dossi et al., illustrates how to perform MEA recording of epileptiform activity generated by human brain obtained from human operative specimens. The authors use a different model of ictogenesis (low-magnesium instead of 4AP) and there is no description of electrical modulation.

Thus, to the best of our knowledge, the experimental method described here does not overlap with any previous methodological paper by other authors. Nonetheless, the paper by Dossi et al. has been cited in the Discussion of our manuscript, to make a comparison with state-of-the-art work.

1. **Here, the authors propose customized recording chambers, long term recording, stimulation processes and show a custom Matlab routine to record and stimulate slices and comparison between mice and rats hippocampal slices. In the abstract the authors do not mention the comparison between mouse and rat brain slices, which occupies a long paragraph (the longest) in the results. This should be added.**

The reviewer is indeed right. This is an important aspect to report that we have overlooked. We now mention the advantages of mouse *versus* rat brain slices in the abstract.

1. **Moreover, the paragraph on the comparison between mouse and rat slices in the results should be shortened and more emphasis should be given to the last paragraph on modulation of ictal activity.**

This is also a good point. As the take-home message is “how to perform electrical neuromodulation in the 4AP *in vitro* model of ictogenesis”, we have shortened the paragraph comparing the performance of brain slices obtained from the two rodent species and we have given more emphasis to the representative results of periodic stimulation at 1 Hz. To this end, some sentences including quantifications were moved from the main text to the corresponding figure legends.

1. **The use of a customized MEA chamber is one of the peculiar features of this methodological paper. However, no details are given on that. Do they just use a smaller ring glued to the MEA to reduce ACSF volumes? Is there any other modification? Which is the diameter of the recording chamber the authors mount on MEA? It should be specified, to understand the difference with commercially available MEA with ring already mounted. A photo of the MEA with the customized recording chamber and a slice in it would be useful (maybe to add in Fig. 2).**

The reviewer is right and we thank him/her for having pointed at this. We have added a figure dedicated to showing all the custom equipment used for this protocol.

1. **The signals obtained with this modified chamber could be compared with commercial ones.**

This is a good point, but feel that detailing this aspect in depth, for example by adding a figure, is a bit out of the scope of the present work. We have added a sentence in the Discussion to mention that the signals are similar in terms of pattern (see for example Boidoet al. *Cortico-hippocampal hyperexcitability in synapsin I/II/III knockout mice: age-dependency and response to the antiepileptic drug levetiracetam. Neuroscience.* **171** (1), 268-283, 2010. doi:10.1016/j.neuroscience.2010.08.046); the substantial difference stems in the fact that the low-volume/laminar flow custom chamber allows increasing the brain slice thickness, which is crucial to achieve a fair connectivity for electrical neuromodulation protocols. In fact, in our hands, the experimental protocols described in this work could be hardly pursued when using the default ring chamber provided with commercial MEAs, as the brain tissue was dying within a very short time.

1. **The Matlab routine should be described deeper. What does is allow exactly? How does it differ from Multichannel systems softwares?**

Thanks for the comment. Our user-friendly GUI represents a quick, simple and flexible tool to map the electrodes of different brain structures. Differently from commercial software usually available with the recording system (e.g. MCS), it is possible to select two different MEA layouts or add custom layouts even with basic MATLAB programming skills. Since we analyzed data using a custom software programmed in MATLAB, it is convenient to have data in the same format and manage the entire process of the analysis within a single software environment, which allows to include additional and more complex algorithms. We have modified the Discussion according to the reviewer’s suggestion.

1. **In the discussion the authors should confer more on the utility of their recording stimulation protocol to study and implement deep-brain stimulation approaches to treat TLE; furthermore, a paragraph on the possible combination of their technique with other techniques (such as imaging) to study ictal activity from different points of view (i.e. Ca2+ signals in different cell types during ictal activity and stimulation) should be added.**

The Discussion has been completely restructured to meet the Journal’s requirements. We have also discussed more in depth the aspects suggested by this point, which undoubtedly add value to the manuscript and also emphasize the relevance and versatility of the MEA recording technique for Neuroscience research.

1. **I did not get which kind of holding chamber was used.**

The reviewer is right. We describe a holding chamber but we have overlooked showing how it looked like. The holding chamber is entirely custom-made using common laboratory glassware and supplies. We have added a figure dedicated to showing all the custom equipment used for this protocol. Thanks for pointing at this.

1. **The amplitude scale in Figure 2 B to E should be shown more clearly.**

Thanks, we did not realize that some scale bars were actually quite concealed by the electrophysiology traces. We hope they are clearly visible now.

1. **Is there any amplitude difference in seizures induced in rats vs mice slices? Is there any difference in the duration during which ictal discharges are still produced by the tissues (without stimulation)?**

The points are interesting but we feel that adding this type of analysis, although it may enrich an original research article, is out of the scope of the present manuscript, which is methodological. Here, we intend to (1) bring about the advantages of using mouse rather than rat brain slices for MEA experiments, here specifically with regard to this model of acute ictogenesis; (2) provide useful information to pursue successful neuromodulation experiments using brain slices coupled to MEA. In addition, the amplitude of field potentials depends on the angle of the recording electrode relative to the field. In fact, the amplitude of field potentials is known to decrease significantly in the proximity of polarity reversal (see for example Leung L.S. (2011) *Field Potential Generation and Current Source Density Analysis.* In: Vertes R., Stackman Jr. R. (eds) Electrophysiological Recording Techniques. Neuromethods, vol 54. Humana Press, Totowa, NJ). Thus, comparison of the amplitudes of ictal discharges generated by brain slices obtained from the two species is inherently biased by the electrode position relative to the field, which cannot be precisely controlled. Finally, the persistence of ictal activity in mouse versus rat brain slices is illustrated in Figure 3F, whereas in the main text we report that rat brain tissue provides a significantly lower experimental output. As we have experienced much difficulty in obtaining reliable recording and stimulation experiments from rat brain slices, we deemed important to provide such information to those who are approaching this experimental paradigm, so that they are aware that the protocols described in this manuscript are quite hard to pursue with rat brain tissue. We also report that this is a known issue, as also mentioned in the cited review by Avoli et al. (*Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro.* Prog Neurobiol. 68 (3), 167-207, 2002)

1. **May the authors discuss a bit differential connection between the subiculum and the parahippocampal structures which may, at least partially account for differences in stimulation results.**

Thanks for pointing this out. We have mentioned this aspect in the Representative Results (*The smaller amount of current required to evoke population responses in mouse versus rat brain slices suggests that the former present with denser connections and are therefore more likely to respond better to sustained electrical neuromodulation*) and in the Discussion (*the use of rat brain tissue proved more challenging, reasonably due to the weaker connectivity as compared to brain tissue obtained from mice*), where we also refer to previous observations reported by Avoli et al. (*Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro.* Prog Neurobiol. 68 (3), 167-207, 2002).

**MINOR CONCERNS:**

1. **l. 72-73: "Modulation of epileptic limbic networks by electrical deep-brain stimulation (DBS) is a valid alternative when pharmacological treatment or neurosurgery are not suitable." This is not yet the case. Please provide a reference or remove the sentence.**

The sentence was rephrased.

1. **L. 90-92: "conventional extracellular field potential recording, where only a few glass pipettes can be accommodated on the brain slice surface due to spatial restrictions." Tungsten wire electrodes are much smaller and should be discussed.**

We partially agree with the reviewer. Indeed, it is not possible to have a similar number of recording electrodes with conventional electrophysiological techniques; as small as they may be, conventional glass pipettes or tungsten electrodes are usually mounted on bulky magnetic stands fixed around the recording chamber. Cross-talk between electrodes is also an issue. In addition, MEA chips offer the advantage of known electrode spacing, which is helpful to trace signal propagation. Since the reviewer makes an interesting point, in order to avoid confusion to the reader, we have rephrased the sentence.

1. **l. 151 and 157: are the authors sure they use a 15 mm Petri dish? I would say 15 cm to keep the MEA chamber inside.**

Yes, 15 cm, thanks. We have corrected the mistake.

1. **l. 160: is really necessary to coat the MEAs overnight with poly-D-lysine for acute slice recording? A shorter treatment before experiment is enough, especially because a slice holding anchor is used anyway to keep the slice in place during recording.**

We partially agree with the reviewer, out of our own experience.

* We have tried coating the MEA for as short as 3h, but the brain slice tended to float.
* MEA chips become contaminated by tissue debris with use, which worsens the signal-to-noise ratio.

Thus, we recommend cleaning and recoating the MEA chips routinely. This can be done the day before the experiment with minimum time investment: enzymatic cleaning and coating take 5 minutes of work each and the waiting time between cleaning and coating can be allocated to other tasks. How often to perform these steps depends on how intensely the MEA chips are used. We find that they are good for 5-6 experimental days at the most. We have added this note in the protocol.

1. **l. 163: is it really necessary to store the MEAs submerged in distilled water? Some water inside the recording chamber would be enough to keep the surface hydrophilic.**

The reviewer is right, but we consider less time consuming and more practical to store all the MEAs in a beaker filled with distilled water.

1. **l. 274: replace "poor" with "pour".**

Again, thanks for pointing at the typos. We have corrected it.

1. **l. 457: why did the authors use the "interval of occurrence" instead of frequency of ictal events given that the ictal duration is not changed?**

The interval between events is measured from-start-to-start. Thus, the frequency equals the inverse of the interval. Maybe the reviewer refers to the seizure-free time?

1. **l. 540: The observation time used to quantify the total ictal activity duration has to be specified.**

In the Representative Results we refer to the paper by Panuccio et al. (Exp Neurol, 2013), where this measurement strategy was used in order to avoid any possible bias due to the occurrence of one single ictal discharge during electrical stimulation, against the numerous ones observed in control (no stimulation) condition. Since the cited paper is currently available through *green* open access only, we agree with the reviewer that this information should be made handier to the reader. Thus, we have added an explanation of how the parameter is calculated and why we prefer it to the typical measurements of duration and interval.

1. **Figure 2 needs to be improved. The photo in panel A (left) is not straight (electrode lines are not completely horizontal) and the scale bar is not well visible. Also traces in panels B-E need to be replaced with higher resolution traces; the grey and white bars cited in the legend of the figure are not really well distinguishable (they both seem grey). The same for Figure 3, panels A and B.**

Thanks, this is a fundamental detail. We have straightened the slice image and we have added dashed lines to help the reader visually link the slice-MEA picture to the traces. The poor resolution is a side-effect of the online pdf conversion.

1. **l. 568: remove the space between "mice" and "1".**

Again, thanks. The typo was corrected.

**Reviewer #2:**

**Manuscript Summary:**

Planar Multi-Electrode Arrays (MEAs) are a mature tool for Neuroscience investigations. However, the requirement of technical skills to manage the big amount of generated data and to run analyses has prevented a widespread application of MEAs. In recent years, the big efforts of Engineers, following the suggestions of the users, made MEAs more and more easy to use by everyone.

Panuccio and colleagues present a detailed procedure on how to set up and perform MEAs recordings of acute brain slices from mice and rats in a model of in vitro seizures induced by 4-AP. They also show ictal-like events recorded in different regions of the temporal lobe and how it is possible to transiently stop ictal-like events with a 1 Hz stimulation.

The draft is easy to read and every protocol step is explained and justified and put in relation to the literature. This way the reader, further than follow the protocol, can understand the reason of each gesture and eventually introduce modifications to the protocol to fit his needs. Moreover, the Authors presented a custom-made program aimed at helping in the selection of the best region of the tissue to stimulate. They also proposed a new design for the chamber around the MEA conceived to minimize the solution in contact with the brain slice, getting a quicker solution exchange and, so, oxygenation of the tissue.

Unfortunately, the draft, as it is, lacks of clarity in some aspects and requires careful editing and extra information.

**MAJOR CONCERNS:**

1. **The custom-made software introduced here is not clearly explained. It is not evident to what extent the program can help in the definition of the best region to stimulate. Is it a graphical summary/notebook about the tested electrodes and their success/failure in eliciting a nice response in the tissue? Is it anything different? It is important to explain the purpose of this software and which is its availability (free, open source, available upon request..?).**

Thanks for the comment. The custom-made software allows us to select the electrodes that correspond to specific structures of the brain slice. This is useful for two reasons:

1. During the experiment, in order to select the electrodes for the electrical stimulation
2. During the offline-analysis. Since we use a custom software developed in MATLAB to analyze the data, it is crucial to have the information regarding the position of the electrodes relative to the slice already available in MATLAB format.

The script mapMEA.m is available upon request. Thanks for pointing at this fundamental detail. We have added a statement in the Introduction, whereas the contact information to request it is in the Acknowledgements.

1. **I found inappropriate the claim of the Authors in the abstract: 'Whereas surgical ablation of the epileptogenic tissue may ameliorate the patient's condition, it also carries significant side-effects'. A concept further highlighted in the introduction without any reference in support of it. On the contrary, the surgical treatment is probably the best therapy for drug-resistant patients. The outcome is mainly positive, which a high percentage of seizure free-patients after the surgery and a low percentage of surgery-related complications (see D'Orio et al., Seizure, 2017; Cardinale - Lo Russo, Epilepsia, 2013 as ones out of the many examples). Deep-brain stimulation (DBS) is also useful as like as it is the information gathered by MEAs recordings to improve it, but the sentences on the surgery 'side-effects' cannot be used as a further justification for the usage of DBS/MEA approaches.**

We partially agree with the reviewer. However, since the scope of this manuscript is to describe a methodological approach, we have rephrased the sentence to avoid any debate that may distract the reader.

1. **Multichannel Systems (MCS) is, of course, one of the best factories for MEAs production. It not only provides high quality chips with a variety of layouts, but it is also available to customize them upon the needs of the users. MCS has been, to my knowledge, the first, and maybe it is still the unique factory selling chips without the chamber. As a consequence, the Authors correctly refer to MCS in many occasions in the draft. I would foster, anyway, the citation of other MEA factories/manufacturers to give more choice (when possible) to the readers and as a matter of fairness.**

The reviewer is indeed right and we realize that our description was restricted to the MCS devices because those are the ones that we use. However, the Scientific Review Editor reminded that JoVE cannot publish manuscripts containing any kind of mention to commercial sources. As we were asked to remove any mention to MCS and other companies from the main text, unfortunately, we cannot mention other factories. MCS is now only mentioned in the Materials Table.

1. **The Authors claimed that their protocol allows for preserving viable acute brain slices in a condition of in vitro epilepsy over a long time. Although I have no doubt on the truthfulness of the claim, I think it would be beneficial to the manuscript to show a plot of at least one of the quantified parameters of the ictal/interictal-like events in a long time interval (hours).**

The purpose of this manuscript is to illustrate how to maintain the viability of rodent brain slices in order to be able to perform prolonged stimulation sessions, e.g. to compare the effects of several stimulation protocols. As prolonged brain slice viability is a pre-requisite to pursue several stimulation sessions, e.g., to compare different stimulation paradigms within the same brain slice, we agree with the reviewer that an overview of a representative long-term recording in the absence of stimulation (control condition) should be shown. Thus, we have added a figure (Fig. 6) to this purpose. Thank for pointing at this.

1. **The quality of the figure is not excellent. It might be due to some pdf settings, so, out of the responsibility of the Authors, but, if it is not the case, please, make sure there are not white shadows on the back of the electrophysiological signals and that the pictures of the MEA + brain slices are correctly aligned to the page.**

Indeed the figure quality is a problem with the pdf files. We have uploaded high-resolution images as per the journal’s guidelines and these can be downloaded separately. With regard to proper alignment, we have checked and corrected any issue. Thanks.

**MINOR CONCERNS:**

1. **The Authors proposed MEAs as provided with a higher 'spatiotemporal resolution' with respect to conventional extracellular recording techniques. Although it is evident to the me that they wanted to highlight the possibility to monitor the activity of the whole brain slice at the same time, it might be misleading for readers not familiar with MEA recordings. I suggest to be more precise in this regard.**

Thanks for the observation. We have modified the text in order to avoid misleading those readers unfamiliar with MEA recordings.

1. **MEA chips, as they come from the manufacturer, are hydrophobic. The protocol described here is to make the surface hydrophilic so that coating and brain slice can adhere. The protocol takes some time to be performed and it guarantees best condition to couple the brain slice to MEA, but people using MEA chips know that, once the chip has made hydrophilic (by keeping it in water or just by using it several times) the coating is not mandatory to get a good MEA/brain slice coupling. This aspect should be mentioned in the manuscript.**

We partially agree with the reviewer, out of our own experience.

* We have tried coating the MEA for as short as 3h, but the brain slice tended to float
* MEA chips become contaminated by tissue debris with use, which worsens the signal-to-noise ratio.

Thus, we recommend cleaning and recoating the MEA chips routinely. This can be done the day before the experiment with minimum time investment: enzymatic cleaning and coating take 5 minutes of work each and the waiting time between cleaning and coating can be allocated to other tasks. How often to perform these steps depends on how intensely the MEA chips are used. We find that they are good for 5-6 experimental days at the most. We have added this note in the protocol.

1. **The comparison between brain slices from mice and rats is a plus of this draft. However, the Author should emphasize in the text the use of MEA recordings of human brain tissue from surgeries on epileptic patients, which is fast growing field of investigation.**

Indeed, and it is a fundamental tool in epilepsy research. We have given more emphasis to this throughout the text.

1. **There is not one picture of the ring/chamber used in the protocol. It is one of the improvements introduced by the Authors and so it would deserve a higher focus on it. Also, it would be nice to show the setting of the custom-made reference in the chamber.**

The reviewer is right and we thank him/her for having pointed at this. However, we do not use any custom reference electrode. We buy saturated KCl pellets from commercial sources, which can be easily assembled onto a 1 mm pin fitting the grounding hole in the MEA amplifier’s head. We have added a figure dedicated to showing all the custom equipment used for this protocol.

1. **At line 367 the Authors wrote that a saturated KCl pellet is the best reference to use. Could they explain why this should be preferable to alternative choices, for instance a silver chloride (Ag-AgCl) reference?**

The reviewer is right. We used the wrong wording. A saturated KCl pellet is actually more practical because it’s ready to use without need to be chlorinated. Thanks for pointing at this. We have modified the sentence.

1. **At line 374 is written that brain slices can be moved over the MEA in order to get the best match. It would be useful for the readers to know which is a suitable approach to do it. A small paintbrush might be appropriate?**

This is a good point. We have added a sentence in the protocol description, which also refers to a new figure illustrating the custom equipment used (e.g., the recording and holding chambers).

1. **Line 375: the reported anchor is a standard one?**

Actually, this is an important detail to stress. Unfortunately, the custom chamber design does not accommodate standard commercial anchors, since they are either too large or too long or too light given their small size compared to the brain slice. Although it may be possible to shorten long ones, this solution is too expensive and time-consuming. Moreover, the modified anchor becomes too light to reliably hold the brain slice at the bottom of the chamber, not guaranteeing a fair electrical contact between the MEA and the brain slice. Thus, we have ordered INOX 316 1 mm-thick bars, which we bend to give them the right shape. We then use nylon threads from stockings to make the mesh, which is glued using cyanoacrylate. The custom anchor is also depicted in the figure showing the custom equipment used for this protocol.

1. **Line 380: please specify which is the best type of microscope to use (stereoscope, inverted).**

We have provided recommendations in the Discussion. This is actually fundamental, since only an inverted microscope makes it possible to visualize the microelectrodes and the brain slice resting on them, which in turn is crucial for electrode mapping. Thanks for pointing at this.

1. **Line 402-403: sampling time and low-pass filter; is it mandatory to use these parameters to run the software? Why?**

It is not mandatory to use the parameters indicated in the text but it is related to the type of analysis. Sampling frequency of 2 kHz allows acquiring field potentials with fair resolution while minimizing hard disc space usage. As an example, a 5-min recording file sampled at 10 kHz takes ~400 MB, but only ~80 MB when the sampling frequency is set to 2 kHz. Higher sampling frequencies may be required, e.g. to record stimulus artifacts or multi-unit activity. To observe field potentials only, we use a live low-pass filter at 300 Hz to cut-off multi-unit activity. Since the decision is not mandatory to run the software, we have decided to remove this information, in order to let the user decide what are the best recording conditions, also depending on the specific analysis he/she wants to perform.

1. **Line 412: single-shock stimulation at 0.2 Hz might still induce neuronal plasticity (see, as an example, Salin et al., PNAS, 1996). 0.1 Hz is more appropriate.**

The referred work by Salin et al. (1996) reported a synapse-specific and reversible induction of plasticity in the hippocampal subfield CA3. In our protocol, stimulation is delivered in the subiculum where we rarely observed changes in the amplitude of the evoked responses, which may be indicative of plasticity phenomena. Moreover, it needs to be emphasized that in the cited work plasticity phenomena were observed following paired pulses delivered at low frequency, whereas our protocol makes use of single-shock stimulation. Further, Salin et al. reported that recovery of evoked responses to baseline condition occurred within a short time of stimulus withdrawal. The electrical modulation protocol described in our manuscript (periodic pacing at 1 Hz) was always performed a few minutes after the I/O curve to avoid any possible bias due to the pulses delivered during the fast I/O.

The observation is relevant in case the experimenter would like to apply stimulation protocols that might be more prone to induce undesired plasticity phenomena. However, this is not the case here. As stated above, the fast I/O performed ‘on the fly’ is very unlikely to induce permanent or long-lasting changes in synaptic function. We feel that discussing this aspect more in detail may only generate confusion in those who are not familiar with such experimental protocols. Rather, we prompt the reader to adjust the inter-pulse interval as most appropriate by suggesting a stimulus frequency of 0.2 Hz **or lower**.

1. **Lines 434-441: Slow and fast interictal-like discharges are introduced. Although it has been reported, in a subsequent paragraph, that the 2 types of these events occur with different frequencies, it is not clear whether there is a difference in the duration of between these events. It would be better to provide a more comprehensive description of both events in this paragraph.**

The main focus of the described protocol is ictal discharge reduction/suppression by a canonical electrical stimulation paradigm; thus we have restricted our analysis to ictal activity. The description of the 4AP-induced epileptiform patterns (including interictal activity) intends to be a qualitative assessment of the reproducibility of this model with MEAs. These patterns have been extensively characterized (see for example Avoli et al. (*Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro.* Prog Neurobiol. 68 (3), 167-207, 2002; Benini, R. & Avoli, M. *Rat subicular networks gate hippocampal output activity in an in vitro model of limbic seizures.* J Physiol. 566 (Pt 3), 885-900, doi:10.1113/jphysiol.2005.088708, 2005). We feel that adding this type of analysis, although may provide further substantiation of the validity of MEA recording, is out of the scope of the manuscript. Indeed, in the Representative Results we do not report detailed quantification of these epileptiform patterns and we rather point the reader to the bar graphs summarizing the effects of periodic stimulation.

1. **Line 460: it is not clear to me how come mice have a higher yield of brain slices versus number of animals than rats.**

The shorter interval of occurrence between ictal discharges generated by mouse brain slices along with the prolonged viability of mouse brain tissue *allows speeding-up the experimental protocols, making it possible to test a greater number of brain slices during a single experimental day*.

**Reviewer #3:**

Manuscript Summary:

This manuscripts describes how to prepare and record epileptiform activity from mouse brain slices using a micro-electrode array. Epileptiform activity is induced using the potassium-channel blocker 4-aminopyridine and the tissue preparation was viable for several hours. This is a very useful technique and the protocol was clearly described. There are some major concerns with the experiment that needs to be addressed.

**MAJOR CONCERNS:**

1. **The introduction discusses the side effects that may occur after surgical resection and suggests that electrical stimulation may be a more viable alternative but yet they use 4-aminopyridine, rather than electrical stimulation, to induce epileptiform activity. As such it is not clear why they proceed via this route rather than performing just electrical stimulation to induce the desired activity.**

The reviewer is indeed right. As electrical stimulation may become a “viable alternative to surgical resection” of the epileptic focus, here periodic electrical stimulation is used to abolish/prevent seizure-like activity (like DBS). Perfusion of brain slices with the convulsant drug 4-aminopyridine is an established model of *in vitro* ictogenesis (see for example Avoli, M. et al. *Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro.* Prog Neurobiol. 68 (3), 167-207, 2002) and it is used here to induce the typical recurrent epileptiform patterns. Indeed, the objective of this manuscript is to illustrate the feasibility and the validity of the MEA recoding technique to study DBS protocols for epilepsy treatment. Thus, we use an in vitro model of acute ictogenesis and we perform periodic electrical stimulation to modulate seizure generation, as in Barbarosie, M. & Avoli, M. *CA3-driven hippocampal-entorhinal loop controls rather than sustains in vitro limbic seizures.* J Neurosci. 17 (23), 9308-9314, 1997.

1. **Curiously, in addition to the use of 4-aminopyridine, they also stimulate electrically and this seems to be overkill as 4-aminopyridine is more than sufficient to induce epileptiform activity as the authors and several others have shown.**

Like explained above, 4-aminopyridine is used to induce epileptiform activity, whereas electrical stimulation is used to modulate the epileptiform pattern induced by 4-aminopyridine. Translating this paradigm to the clinical setting, 4AP-treated brain slices would represent the epileptic patient, whereas electrical stimulation would represent DBS for epilepsy treatment.

1. **Lastly, their method touts as an advantage the ability to record for several hours. Again this seems to be overkill as the seizures only last several seconds and it is not clear why hours of recordings is really necessary to obtain reproducible findings.**

The prolonged (hours) of recording may be required, for example, in case the experimenter wants to compare several stimulation protocols, which must be therefore performed in the same brain slice for statistical robustness. In our hands, stimulation phases may take 20-45 minutes. When testing 3 stimulation protocols, each preceded by a control phase and followed by a recovery phase, the experiment may last 3-5h. Moreover, one may wish to analyze network features in the long-term, hence record activity for several hours and see if any change in any feature of interest occur. This is not uncommon and our manuscript derives from our own experience and belief that having some good reference on how to make this happen comes in really handy to those who wish to study DBS protocols *in vitro* using MEAs. However, like the reviewer also pointed out, it is not of course mandatory to perform hours-long experiments; rather, having the right experimental conditions to do so if need be is undoubtedly an advantage. We thank the reviewer for having raised this important point. We have added two sentences in the Introduction to bring some examples of the usefulness of maintaining a good brain slice viability for several hours.

1. **For the preparation of the MEA, glass rings are not used. How are liquids contained around the slice? There is no picture of this assembly, only a picture of the slice atop of the MEA.**

We use a custom recording chamber and we thank the reviewer for having highlighted the lack of sufficient detail in this regard, since the custom recording chamber is what makes the described experimental protocol possible. As also required by the other reviewers, in the revised version of the manuscript we add more details on this custom chamber, including a dedicated figure. Section 1 of the protocol provides instructions on how to assemble the recording chamber onto the MEA chip. The section is also highlighted in yellow for the narrator.

**MINOR CONCERNS:**

1. **In the results section, there is mention of the disadvantageous condition that is intrinsic to MEA recordings but there is no mention of what this condition is.**

The submerged chamber and the fact that the brain slice lies onto a piece of glass affect tissue oxygenation. Please, refer both to Introduction and Discussion, which stress these aspects more in detail.