Journal of Visualized Experiments

Recording and Modulation of Epileptiform Activity in Rodent Brain Slices Coupled to Micro Electrode Arrays

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57548R2
Full Title:	Recording and Modulation of Epileptiform Activity in Rodent Brain Slices Coupled to Micro Electrode Arrays
Keywords:	epilepsy; 4-aminopyridine; brain slice; mouse; multi-electrode array; neuromodulation
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Additional Information:	
Question	**Response**
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DI TECNOLOGIA

To Dr Alisha DSouza, Review Editor of JoVE
Cc Dr Ronald Myers, Science Editor of JoVE

Genova, January 26th, 2018

*Re: Submission of the revised version of the manuscript entitled "**Recording and Modulation of Epileptiform Activity in Rodent Brain Slices Coupled to Micro Electrode Arrays**" by Gabriella Panuccio, Ilaria Colombi and Michela Chiappalone.*

Dear Editors,

Following your kind invitation to submit a manuscript for 'The Journal of Visualized Experiments' (JoVE), herewith enclosed please find our contribution. This is a revised version of the previously submitted paper, which includes all the changes required both by the Scientific Review Editor and the Scientific Reviewers.

Together with the manuscript (which includes all the performed changes, highlighted in blue), you can find a rebuttal letter, where we have addressed point-by-point the indications of the Scientific Review Editor as well as the Scientific Reviewers' concerns. The current version of our manuscript has substantially improved thanks to the constructive comments and observations received. We hope that you will find the new version suitable for publication in the Journal.

On behalf of all the authors, I state that the whole manuscript has not been and will not be submitted elsewhere for publication.

Yours Sincerely,

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TITLE:

Recording and Modulation of Epileptiform Activity in Rodent Brain Slices Coupled to Microelectrode Arrays

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KEYWORDS:

Epilepsy, 4-aminopyridine, brain slice, mouse, multielectrode array, neuromodulation

SUMMARY:

We illustrate how to perform recording and electrical modulation of 4-aminopyridine-induced epileptiform activity in rodent brain slices using microelectrode arrays. A custom recording chamber maintains tissue viability throughout prolonged experimental sessions. Live electrode mapping and selection of stimulating pairs are performed by a custom graphical user interface.

ABSTRACT:

Temporal lobe epilepsy (TLE) is the most common partial complex epileptic syndrome and the least responsive to medications. Deep brain stimulation (DBS) is a promising approach when pharmacological treatment fails or neurosurgery is not recommended. Acute brain slices coupled to microelectrode arrays (MEAs) represent a valuable tool to study neuronal network interactions and their modulation by electrical stimulation. As compared to conventional extracellular recording techniques, they provide the added advantages of a greater number of observation points and a known inter-electrode distance, which allow studying the propagation path and speed of electrophysiological signals. However, tissue oxygenation may be greatly impaired during MEA recording, requiring a high perfusion rate, which comes at the cost of decreased signal-to-noise ratio and higher oscillations in the experimental temperature. Electrical stimulation further stresses the brain tissue, making it difficult to pursue prolonged recording/stimulation epochs. Moreover, electrical modulation of brain slice activity needs to target specific structures/pathways within the brain slice, requiring that electrode mapping be easily and quickly performed live during the experiment. Here, we illustrate how to perform the recording and electrical modulation of 4-aminopyridine (4AP)-induced epileptiform activity in

rodent brain slices using planar MEAs. We show that the brain tissue obtained from mice outperforms rat brain tissue and is thus better suited for MEA experiments. This protocol guarantees the generation and maintenance of a stable epileptiform pattern that faithfully reproduces the electrophysiological features observed with conventional field potential recording, persists for several hours, and outlasts sustained electrical stimulation for prolonged epochs. Tissue viability throughout the experiment is achieved thanks to the use of a small-volume custom recording chamber allowing for laminar flow and quick solution exchange even at low (1 mL/min) perfusion rates. Quick MEA mapping for real-time monitoring and selection of stimulating electrodes is performed by a custom graphic user interface (GUI).

INTRODUCTION:

Epilepsy is a life-threatening progressive disorder causing uncontrolled activity of the brain[1]; it carries among the highest burden of disease and significant social stigma[2,3]. TLE is the most frequent syndrome (40%) and the most frequently (~ 30%) resistant to anti-epileptic medications[4]. While surgical ablation of the epileptogenic tissue may ameliorate the patient's condition, it is not feasible in all patients and may not guarantee a completely seizure-free life[5]. Modulation of epileptic limbic networks by electrical DBS is a promising approach when pharmacological treatment or neurosurgery are not suitable.

Rodent brain slices are a valuable tool to study how neuronal networks function in health and disease[6] by means of *in vitro* electrophysiology techniques, as they preserve, at least in part, the original architecture and connectivity of the brain region(s) of interest (ROI). In particular, horizontal combined hippocampus-entorhinal cortex (hippocampus-EC) slices comprise the essential neuronal networks involved in TLE and are therefore routinely employed in *in vitro* TLE research[7].

Seizure-like activity may be acutely induced in brain slices by changing the ionic composition of the artificial cerebrospinal fluid (ACSF), such as decreasing magnesium while increasing potassium[8-10], or by means of pharmacological manipulations, such as blocking inhibitory GABAergic activity (see[11] for a comprehensive review). However, these models are based on the unbalanced alteration of excitation and inhibition; thus, they do not allow studying the interaction and concerted contribution of excitatory and inhibitory networks to ictogenesis. Continuous perfusion of brain slices with the convulsant drug 4AP enhances both excitatory and inhibitory neurotransmission, thus allowing to study acute ictogenesis while keeping synaptic activity overall intact[12].

MEAs allow recording the electrical activity generated by neuronal networks from a greater number of observation points as compared to conventional extracellular field potential recording, where spatial restrictions limit the number of electrodes that can be accommodated on the brain slice surface. Moreover, MEA chips offer the added advantage of known inter-electrode distance, which is very useful to trace propagation and assess the traveling velocity of recorded signals. Although initially conceived for recording from cultured neurons[13,14], MEAs are now also used to characterize the electrophysiological features of acute brain slices obtained from rodents[15] and humans[16]. Thus, in the context of epilepsy research, MEAs represent a

valuable tool to pinpoint neuronal networks' interactions at the core of ictogenesis[16-18].

However, MEA recording inherently carries technical challenges in obtaining or maintaining a stable epileptiform pattern throughout long (several hours) experimental protocols. First, tissue oxygenation may not be adequate within the large and round submerged-type recording chamber[19,20] typical of commercially available MEAs, while poor signal-to-noise ratio and temperature instability might affect the quality of the recording when a high perfusion rate (6–10 mL/min) is used to improve the oxygen supply to the brain slice (see for example the technical notes on heating and perfusion equipment[21]). Second, recurrent discharges featuring high frequency components, such as epileptiform discharges, are hardly observed when using submerged-type recording chambers[19]; this is particularly the case when the acute epileptiform pattern is chemically induced and involves ephaptic mechanisms, as it is the case for the 4AP model[22] (see[11] for a comprehensive overview). To overcome these limitations, several strategies have been proposed by researchers. For example, increasing the perfusion rate[19,20] while reducing brain slice thickness (≤ 300 µM) and area[17,18] are crucial factors to achieving adequate oxygen supply to the tissue. In addition, improved brain slice viability can also be accomplished by using perforated MEAs (pMEAs), which allow perfusing the brain tissue from both sides[18].

Whereas the described approaches have significantly improved the feasibility of MEA recording from brain slices, they have not been tested against prolonged (several hours) recording and electrical stimulation sessions, the latter representing a significant stress for the brain tissue. Prolonged recording sessions may be required to study the evolution in time of specific features of epileptiform patterns that would not be possible to unmask by short-term measurements. In the context of DBS research, prolonged experimental protocols may be required to evaluate and compare the effects of several stimulation paradigms in the same brain slice.

When only spontaneous electrical activity needs to be recorded, mapping of the electrodes with respect to the ROI is usually done *a posteriori*, *i.e.*, during data analysis; instead, the study of evoked responses or of electrical neuromodulation paradigms requires that stimulation be delivered to specific ROI(s), dictating the need of quick and easy live electrode mapping during the experiment.

Here, we illustrate a simple experimental protocol that allows for induction and maintenance of a stable 4AP-induced epileptiform pattern in adult rodent brain slices. The observed activity faithfully reproduces the electrophysiological features of this model as characterized using conventional extracellular electrophysiology techniques. It persists for several hours and outlasts sustained electrical stimulation for repeated prolonged epochs. The chambers used to maintain and incubate the brain slices can be easily assembled using standard laboratory supplies (**Figure 1A**), whereas a custom recording chamber allowing for optimal solution exchange rate and laminar flow (**Figure 1B**) can be obtained from commercial sources or using 3D printing technology at an affordable price. Quick mapping of the ROI(s) for real-time monitoring and for the selection of stimulating electrodes is made possible by a custom user-friendly GUI named **mapMEA**, freely available upon request.

[Place Figure 1 here]

PROTOCOL:

All methods described here have been approved by the Italian Ministry of Health (authorization n. 860/2015-PR) in compliance with the EU directive 2010/63/EC on animal welfare.

1. Preparation of the MEA/Custom Chamber Assembly

Note: Start 2 days before the experiment. Use a MEA without a ring (see **Materials Table**).

1.1. **Clean the MEA (duration: 35–40 min).**

1.1.1. Dissolve an enzymatic cleaner powder into warm distilled water. Brush the warm cleaning solution onto the MEA surface then soak the MEA into the warm cleaning solution for at least 3 h.

1.1.2. Rinse the MEA thoroughly with distilled water and dry it using a lint-free no-scratch tissue.

1.2. **Assemble the MEA with the recording chamber (duration: 10 min + overnight).**

1.2.1. Evenly spread the elastomeric sealant on the bottom surface of the recording chamber. Make sure there are no bubbles.

1.2.2. Mount the recording chamber onto the MEA with the help of tweezers and apply gentle pressure. If there are any bubbles, slightly move the chamber in a circle while applying gentle pressure with fingers until all the bubbles have disappeared. Spread a sealant layer all the way around the outer border of the recording chamber.

Note: **CRITICAL!** Do not cover the MEA contacts with the sealant.

1.2.3. Place the MEA/chamber assembly inside a 15 cm Petri dish. Place a 5 mL Petri dish or a 10 mL beaker filled with distilled water close to the MEA. Cover everything to maintain a moist environment. Let cure at room temperature overnight.

Note: The protocol can be paused here.

1.3. **Coat the MEA to make it hydrophilic (duration: 5–10 min + overnight).**

1.3.1. Place the MEA into a 15 cm Petri dish. Pour 50 µL of poly-D-lysine onto the MEA recording area. Close the Petri dish, seal it with sealing film, and store overnight at 4 °C.

1.3.2. Rinse the MEA thoroughly using distilled water and store the MEA at 4 °C in distilled water.

Note: The protocol can be paused here. Coated MEAs may be stored and reused for several

experiments. Routinely cleaning and re-coating the MEA helps remove tissue debris and improve signal-to-noise ratio.

2. Preparation of Stock Solutions

Note: Start 1 day before the experiment (duration: ~2 h). Stock solutions help speed-up the experiment on the experimental day. They can be prepared in advance and stored. See **Table 1** for concentration factor and composition. Refer to **Table 2** for composition of final solutions.

2.1. Dissolve the chemicals in distilled water at room temperature. Filter the stock solutions with a filter bottle (filter diameter: 0.22 µm).

2.2. Store at 4 °C (see **Table 1** for recommended maximum storage time).

Note: The protocol can be paused here.

3. Preparation of Agar

Note: Start 1 day before the experiment (duration: ~1 h).

3.1. Pour 250 mL of distilled water into a 500 mL beaker and start stirring at 350 rpm. Add 5 g of agar and wait until it is completely dissolved.

3.2. Heat the solution at 250–300 °C. Let the water evaporate until the solution is ~200 mL to yield a 2.5% w/v agar solution.

Note: The temperature should be high enough to let water evaporate without bubbling.

3.3. Pour the agar solution onto a 200 mL plastic square box of ~1.5 cm-high walls and let it cool down at room temperature until it becomes solid.

3.4. Cover the box and store it at 4 °C. The agar block is good for several weeks.

Note: The protocol can be paused here.

4. Preparation of the Frozen Plane

Note: Start 1 day before the experiment.

4.1. Fill a flat-bottom stainless steel bowl with distilled water up to 0.5–1 cm below the rim.

4.2. Store at -20 °C overnight.

5. Preparation of Neuroprotective Cutting ACSF

Note: Start 1 day before the experiment or the same day of the experiment (see **Table 1** and **Table 2**) (duration: 30 min).

5.1. Pour 200 mL of distilled water in a 500 mL beaker and start stirring using a magnetic stirrer at 350 rpm.

5.2. Add 50 mL of stock B and 50 mL of stock C (see **Table 1**).

5.3. Add 3 mM pyruvic acid, 208 mM sucrose, 10 mM D-glucose, and 1 mM L-ascorbic acid.

Note: The actual volume of pyruvic acid depends on the density and purity and may vary with the batch. Always calculate the required volume when opening a new batch.

5.4. Cover with sealing film and let stir for 30 min.

5.5. Transfer the solution to a 500 mL volumetric flask and add distilled water to fill the flask.

5.6. Seal the volumetric flask with sealing film and gently invert 3–5 times until the solution is evenly clear.

5.7. Transfer the cutting ACSF into a glass bottle with cap.

5.8. Store the cutting ACSF at -80 °C for 20–30 min or at 4 °C overnight to cool down to 4 °C.

Note: The protocol can be paused here if overnight cooling is preferred. Otherwise the time required for cooling the cutting ACSF may be used to pursue step 6.

6. Preparation of the Holding ACSF

Note: Prepare the solution fresh on the day of the experiment (duration: 5–10 min). The holding ACSF will be used to rinse the brain slices from the cutting ACSF during the slicing procedure (see step 8.16) and for brain slice long-term storage and pre-warming. To rinse the brain slices, 100 mL of holding ACSF is sufficient. The holding and pre-warming chambers used in this protocol are custom-made and contain a volume of 300 mL and 100 mL, respectively (*cf.* **Figure 1A**). With this setting, **500 mL of holding ACSF** is sufficient. Chambers obtained from commercial sources may contain a different volume, in which case the overall amount of holding ACSF to be prepared should be adjusted accordingly.

6.1. Pour 200 mL of distilled water in a 500 mL volumetric flask.

6.2. Add 50 mL of stock A, 50 mL of stock B, 6.5 mL of stock M, and 1 mM L-ascorbic acid. Gently agitate using rotating movements until L-ascorbic acid is completely dissolved.

6.3. Add distilled water to reach the final volume, seal the volumetric flask with sealing film, and gently invert 3–5 times until the solution is evenly clear. Refer to **Table 2** for the holding ACSF composition.

7. Preparation of the Experimental Benches

Note: This requires 15 min, plus 30 min to obtain a steady temperature of the warm bath.

7.1. Recording bench

7.1.1. Start the warm bath, set it to 32 °C, and cover it to maintain its temperature.

Note: The warm bath used in this protocol can be custom-made using a hard plastic box and an aquarium thermostat pre-set at the desired temperature. Steady desired temperature may be reached in ~30 min starting from room temperature. Since the pre-warming and incubating chambers sit at the bottom of the plastic box, the water level should be just enough to cover the thermostat so as to keep the chambers from floating.

7.1.2. Assemble the holding and the pre-warming chambers (*cf.* **Figure 1A**), and pour the holding ACSF into them. Keep the holding chamber at room temperature and place the pre-warming chamber inside the warm bath. Start bubbling the solutions with 95% O_2/5% CO_2 gas mixture and remove any trapped bubbles using an inverted Pasteur pipette. Keep covered.

7.2. Slicing bench and vibratome

7.2.1. Glue a small agar block onto the center of the specimen disc using cyanoacrylate glue.

7.2.2. Put a 250 mL beaker into an ice bucket and pour cutting ACSF into it.

7.2.3. Take two 100 mL beakers and pour 50 mL of holding ACSF into each. Leave the beakers at room temperature. These are the rinsing beakers.

7.2.4. Start bubbling the solutions with a 95% O_2/5% CO_2 gas mixture.

7.2.5. Mount the buffer tray onto the vibratome and surround it with crushed ice. Pour some ethanol on the crushed ice to aid in maintaining cold temperatures during the slicing procedure. After pouring ethanol, add more ice as needed.

Note: A temperature of 2 °C is optimal.

7.2.6. Place the bubbler inside the buffer tray, then assemble and mount the vibratome blade block.

Note: Make sure that the blade is mounted at clearance angle of ~10°.

7.2.7. Put crushed ice in a 500 mL beaker up to 2/3 of the volume and fill with distilled water.

7.2.8. Take the frozen bowl out of the freezer, place it upside-down on the slicing bench, and cover it with a paper towel and a filter paper disc.

7.3. **Anesthesia bench**

7.3.1. Place a small bowl in an ice-filled tray and pour cutting ACSF in it. Start bubbling with a 95% O_2/5% CO_2 gas mixture.

8. Brain Slice Preparation and Maintenance

Note: This protocol makes use of adult (4–6 weeks-old) male CD1 mice, but other strains (*e.g.*, c57/bl6[17,18]) can be used. We also later compare the experimental output obtained using mouse brain slices with that yielded by brain slices obtained from male Sprague-Dawley rats of the same age. The following steps refer to the preparation of **partially disconnected** brain slices in which CA3-driven **fast** interictal-like activity is restrained to the hippocampus proper and cannot propagate to the parahippocampal cortices, as described in[23]. Hippocampus-cortex disconnection is a pre-requisite to pursue electrical modulation studies in using the hippocampus-EC brain slice preparation. The vibratome used refers to the model listed in the **Materials Table**. Other models may require a different procedure.

8.1. Anesthetize the rodent using isoflurane 5% in a carbogen gas mixture delivered to an anesthesia induction chamber at 2 L/min.

8.2. Under deep anesthesia (no reflexes in response to foot and paws pinch), extract the brain within 1 min following standard procedures as in[23]. Place the brain into the small bowl containing equilibrated ice-cold cutting ACSF and let chill for 90–120 s.

Note: Do not let the brain chill for too long, otherwise it may freeze.

8.3. Pour the ice-cold cutting ACSF into the buffer tray. Spread cutting ACSF onto the filter paper placed on the frozen bowl. Place the brain onto the filter paper and isolate the required brain tissue block by removing the cerebellum and cutting the frontal pole out straight.

8.4. With the help of a bent spatula, glue the dorsal side of the brain onto the specimen disc with the cut frontal side facing the agar block and the occipital pole facing the vibratome blade. Use a thin layer of cyanoacrylate glue. Place the specimen disc in the buffer tray immediately and secure it.

8.5. Adjust the sectioning range so as to move the vibratome blade all the way across the tissue up to the agar block. Adjust the specimen disc height to reach the blade level with the brain tissue block.

8.6. Discard the tissue sections until the hippocampus is clearly visible (usually ~ 900 µm). Then, set a slice thickness of 400 µm and start slicing to retain the tissue sections. For each brain section split the two hemispheres and trim the unnecessary tissue to obtain two brain slices. As the buffer tray is heightened during subsequent sections, fill the buffer tray lodgment with iced distilled water (see step 7.2.7).

8.7. Use an inverted Pasteur pipette to gently transfer the brain slices in the first rinsing beaker, empty the Pasteur pipette, and transfer the brain slices to the second rinsing beaker. Then, transfer the brain slices into the holding chamber and let them recover for at least 60 min.

Note: Usually it is possible to obtain 6–8 brain slices overall. The protocol may be paused here.

9. Preparation of ACSF Containing 4-AP (4AP-ACSF)

Note: This requires 10 min for preparation, plus 20 min of warming. **CAUTION!** 4-AP is a convulsant drug and it is toxic. Handle with gloves and avoid spilling.

9.1. Pour 200 mL of distilled water in a 500 mL volumetric flask.

9.2. Add 50 mL of stock A, 50 mL of stock B, 5 mL of stock M, and 1 mM L-ascorbic acid. Gently agitate using rotating movements until the L-ascorbic acid is completely dissolved.

9.3. Add 500 µL of 4AP stock.

9.4. Add distilled water to reach the final volume, seal the volumetric flask with sealing film, and gently invert it 3–5 times until the solution is evenly clear.

9.5. Assemble the 4AP incubating chamber (*cf.* **Figure 1A**) and pour ~80 mL of 4AP-ACSF into it. Cover the chamber, transfer it to the warm bath, and start bubbling with a 95% O_2/5% CO_2 gas mixture. Remove any trapped bubbles using an inverted Pasteur pipette. Keep covered.

9.6. Wait until the 4AP-ACSF temperature is 30–32 °C (~20 min).

Note: The protocol may be paused here if brain slices are still recovering.

10. Pre-warming and Incubation of Slices in 4AP (Duration: 90 min)

10.1. Check that the pre-warming ACSF and the 4AP-ACSF temperature is 30–32 °C.

10.2. Use an inverted glass Pasteur pipette to transfer 1 brain slice into the pre-warming chamber and let the tissue rest for 25–30 min. Then transfer the brain slice into the 4AP-ACSF incubating chamber and let it rest for 60 min.

11. Preparation of the MEA Set-up

Note: Start 15 min before recording.

11.1. Transfer the remaining volume of 4AP-ACSF into a 500 mL Erlenmeyer flask.

11.2. Place the Erlenmeyer flask on a shelf above the MEA amplifier and use tubing to allow the solution to continuously feed a 60 mL syringe. Adjust the height of the Erlenmeyer flask and the syringe to allow a gravity-fed rate of 1 mL/min.

Note: The correct height depends on the tubing inner diameter (ID). For tubing of 5/32 inches ID, 30 cm are sufficient.

11.3. Start bubbling the 4AP-ACSF in the Erlenmeyer flask and in the syringe with a 95% O_2/5% CO_2 gas mixture.

11.4. Let the 4AP-ACSF flow through the perfusion tubing into a beaker until there is no air inside; then stop the solution flow.

11.5. Connect the heating element at the MEA base to the thermostat. Place the dry MEA chip inside the MEA amplifier and secure the amplifier head. Use a plastic Pasteur pipette to transfer 4AP-ACSF to the inlet and outer reservoir of the recording chamber.

11.6. Secure the heating cannula to a magnetic holder and place its tip inside the recording chamber inlet port; attach the magnetic holder to a magnetic strip on the MEA amplifier head; connect the perfusion tubing to the cannula; connect the cannula to the thermostat.

Note: The heating cannula should be covered by beveled polytetrafluoroethylene (PTFE) tubing to reach the recording chamber inlet port and to minimize noise due to the metallic material of the cannula. When the inlet reservoir is adequately filled with 4AP-ACSF, drops falling from the perfusion system should not be visible.

11.7. Place the suction needle inside the reservoir and verify that there is negative pressure by submerging the suction needle into the ACSF; check for a constant low-frequency suction noise.

Note: The suction needle should be placed so as to allow the 4AP-ACSF to flow just above the brain slice surface. A vacuum line or a low-noise vacuum pump can be used.

11.8. Set the flow regulator to allow a flow-rate of 1 mL/min and start perfusing.

Note: Gravity-fed perfusion eliminates the noise that might be caused by peristaltic pumps; if peristaltic pumps are preferred, low-noise models are mandatory.

11.9. Once 4AP-ACSF is flowing through the cannula, turn on the thermostat. Set the heating

cannula to 37 °C and the MEA base to 32 °C to achieve a temperature of 32–34 °C inside the recording chamber.

Note: **CAUTION!** Never heat the cannula without solution in it or it might be irreversibly damaged. Set the temperature of the cannula higher than the recording temperature (*i.e.*, 32–34 °C) to account for the **intrinsic temperature offset** between the set value and actual value at the tip of the heating cannula and within the recording chamber. The flow rate, environment temperature, and volume of the recording chamber all influence the temperature of the recording solution. The settings reported in step 11.9 are optimized for the described protocol and equipment. Always check the actual recording temperature using a thermocouple and adjust the settings as needed. Do not heat the MEA base above 34 °C to avoid overheating the brain slice.

11.10. Place the external reference electrode in the recording chamber inlet reservoir.

Note: Although MEA chips are equipped with an internal reference electrode, this is covered by the custom recording chamber. Thus, an external reference electrode must be used. A saturated KCl pellet is the most practical, since it is ready to use without need for chlorination.

12. MEA Live Mapping

12.1. Once the 4AP-ACSF level and the recording temperature are stabilized as desired, turn the perfusion and the suction stopcocks to the off position to temporarily stop them.

12.1.1. Quickly transfer one brain slice onto the MEA recording chamber using an inverted glass Pasteur pipette. Adjust its position on the MEA recording area as needed using a fire-polished curled Pasteur pipette (**Figure 1C**) or a soft compact small brush. Place the hold-down anchor (**Figure 1D**) on the brain slice. Restart the perfusion and the suction by turning their stopcocks back to the on position.

Note: **CRITICAL!** The slice should be transferred, and the perfusion restarted within 60 s or the tissue might die. The slice hold-down anchor should be kept in 4AP-ACSF to prevent the brain slice from moving while placing the anchor on the brain slice due to differences in superficial tension. The anchor to secure the brain slice onto the MEA can be custom-made using stainless steel wire and nylon thread (**Figure 1D**) or obtained from commercial sources. **Figure 1E** shows the final experimental set-up with the MEA chip connected to the amplifier's head: a brain slice resting on the MEA chip within the recording chamber is held down by the custom anchor. The reference electrode (red circle) and the PTFE tubing covering the heating cannula (red arrow) are positioned in the inlet reservoir, whereas the suction needle (blue arrow) is positioned in the outlet reservoir.

12.2. Take a picture of the brain slice using a camera mounted on an inverted microscope stage.

12.3. Run the script **mapMEA** on the computer software to start the GUI to map the electrodes.

Note: The custom-made software allows the user to select the electrodes that correspond to specific structures of the brain slice. This step is crucial to activate the correct pathway and suppress ictal activity by using electrical stimulation.

[Place Figure 2 here]

12.4. Click the **Browse** button to load the picture of the brain slice. Make sure that the reference electrode appears in the upper row of the left half-side of the MEA (**Figure 2A, triangle mark**). Click the **Activate Pointer** button, then select the top and bottom electrodes in the leftmost row of the array to mark the XY coordinates for image straightening and electrode mapping.

12.5. From the **slice type** drop-down menu select **Horizontal.** Tick the **Default structures** checkbox.

Note: It is possible to customize the structures by selecting the **Enter New Structures** button. The default structures for the horizontal brain slice are depicted in **Figure 2A**.

12.6. Using the numbered pushbuttons below the brain slice picture, select the electrodes corresponding to the ROI and click the corresponding pushbutton in the structures panel to assign them (**Figure 2B**); repeat this step for each ROI.

12.7. Press the **Save** button: the software generates a result folder named **#EXP_LabelledElectrodes** containing a table reporting the selected electrodes and ROIs.

13. Recording and Electrical Modulation of the Epileptiform Activity

13.1. Allow the brain slice to stabilize within the recording chamber for 5–10 min before recording.

13.2. Turn on the stimulus unit at least 10 min prior to the stimulation protocol to allow self-calibration and stabilization. Start the stimulus control software and verify that stimulator and MEA amplifier are correctly connected as indicated by a green LED in the main panel of the stimulus control software. Please refer to the manuals of the specific instruments and software for additional details, *cf.* **Materials Table**.

13.3. Set up the stimulation in bipolar configuration. Select electrode pairs in contact with the pyramidal cell layer of the CA1/proximal subiculum (*cf.* [24,25]) among the ones mapped with the script **mapMEA** (*cf.* section 12). Use a wire to connect one of the selected electrodes to the negative plug of the stimulator and the other electrode to the positive plug of the same stimulator channel. Use another wire to connect the ground of the stimulator to the ground of the amplifier.

13.4. Start the recording software. To acquire data, press the **PLAY** button in the main panel of

the recording software. Record at least 4 ictal discharges.

Note: A sampling frequency of 2 kHz allows acquiring field potentials with fair resolution while minimizing hard disc space usage. A 5-min recording file takes ~80 MB. Higher sampling frequencies may be required, *e.g.*, to record stimulus artifacts or multi-unit activity. To observe field potentials only, use a live low-pass filter at 300 Hz to cut off multi-unit activity.

13.5. Determine the stimulus intensity.

13.5.1. In the stimulus control software, use the main panel to design a square biphasic positive-negative current pulse duration of 100 µs/phase.

Note: **CAUTION!** Direct current stimulation requires a balanced charge pulse to avoid damaging the equipment.

13.5.2. Run a **fast** input/output (I/O) test to identify the best stimulus intensity. Deliver the stimulation pulse designed at step 13.5.1 at 0.2 Hz or lower by adding an inter-pulse interval of 5 s or longer in the appropriate form of the stimulus control software. In the **pulse amplitude** tab enter an initial pulse amplitude of 100 µA/phase and increase by 50–100 µA steps at each trial until stimulation can reliably evoke interictal-like events in the parahippocampal cortices (check the signals visualized by the recording software).

13.6. Electrical modulation of limbic ictogenesis

13.6.1. Program the stimulus unit to deliver the stimulation protocol of interest. Use the stimulus amplitude identified during the I/O test.

Note: The failure rate of evoked responses should be ≤ 20%.

13.6.2. After stimulation stops, verify network recovery to pre-stimulus condition by recording at least 4 ictal discharges (as in step 13.4).

REPRESENTATIVE RESULTS:

Planar MEAs with 6 x 10 layout and 500 µm electrode spacing are the ideal recording device for the experimental protocol described here, since their recording area spans throughout the brain slice in its entirety (**Figure 3A**, see also[17]). Although perforated MEAs (pMEAs) would be preferable to improve tissue oxygenation, their recording area is too small (~2 mm diameter). This does not allow the simultaneous visualization of electrical signals generated by the hippocampus and the parahippocampal cortices (data not shown; see[18]).

The observed 4AP-induced epileptiform pattern (**Figure 3A**) faithfully reproduces what is typically observed in this *in vitro* model using conventional field potential recording and comprises three types of activity[7,26]: (**i**) ictal-like events (**Figure 3B**, arrowheads) are robust long-lasting (> 20 s, range: 20–60 s) events resembling the electrographic features of seizure activity with tonic and

clonic components (**Figure 3C**); they are generated within the parahippocampal cortices every 3–5 min and re-enter the hippocampal formation through the dentate gyrus (**Figure 3D**, arrow); (**ii**) slow interictal-like discharges (**Figure 3B**, EC trace, black bar; expanded in **Figure 3E**) are short (< 1 s) population events generated between ictal events ubiquitously within the hippocampus-EC brain slice preparation and occur at a slow pace (range 10–30 s); (**iii**) fast interictal-like discharges (**Figure 3B**, CA3 trace, black bar; expanded in **Figure 3E**) are recurring brief (< 1 s) population events generated by the CA3 hippocampal subfield; when the Schaffer Collaterals are preserved, fast CA3-driven interictal events propagate along the hippocampal loop and exert an anti-ictogenic effect[24] (data not shown); to prevent ictal activity rundown, for the purpose of the described protocol, CA3 output is disrupted (*cf.* **Figure 3B, E**). It needs to be noted that the fast CA3-driven interictal activity recorded using MEAs occurs at a slower pace (~ 0.4 Hz) than what is observed using conventional field potential recording with glass pipettes (~ 1 Hz, range: 0.5–2.0 Hz, data not shown).

The successful outcome of electrical neuromodulation in rodent brain slices depends on the activation of specific neuronal pathways[27] along with the intrinsic connectivity among the regions included in the preparation[24]. We have tested slices obtained from adult male Sprague-Dawley rats and CD1 mice and we have found that mouse rather than rat brain slices offer the best trade-off among size, thickness, and intrinsic connectivity. First, thanks to their smaller size, they fit the small recording area of commercially available MEAs better (**Figure 4A**, left: rat, right: mouse); second, they are more resilient to the disadvantageous condition that is intrinsic to MEA recording (*cf.* **Introduction**): although ictal-like discharges generated by these two tissues (**Figure 4B**) are of similar duration (**Figure 4C left**), their rate of occurrence is significantly shorter in mouse brain slices (n = 10 slices each species; 2-tailed unpaired t-test, $p < 0.001$; **Figure 4C right**). The latter allows speeding-up the experimental protocols, making it possible to test a greater number of brain slices during a single experimental day: for this set of representative results, we could test a similar number of brain slices from the two species (rat: n = 58; mouse: n = 52), but the number of mice (n = 18) was 2-fold smaller than the number of rats (n = 42).

Moreover, mouse brain slices appear to present with denser connections and are therefore more likely to respond better to sustained electrical neuromodulation (**Figure 4D**). Thus, in this *in vitro* model of ictogenesis, the viability for electrical stimulation aimed at controlling ictal activity is significantly greater for mouse than for rat brain tissue. This aspect is also reflected by the significantly higher success rate of stimulation experiments performed using mouse versus rat brain slices even when pursuing several sustained stimulation protocols (**Figure 4E**). In fact, mouse brain tissue appears to better withstand sustained electrical stimulation as suggested by the higher survival rate within the tested experimental time-frame of 4 h (**Figure 4F**). Thus, the smaller size along with the better preservation of intrinsic connectivity makes mouse brain slices the best candidate to perform MEA recording aimed at studying hippocampal-parahippocampal network interactions and to evaluate electrical neuromodulation protocols that are relevant to TLE.

Periodic pacing delivered in the CA1/subiculum is known to control limbic ictogenesis in the 4AP-treated hippocampus-EC slice preparation[24,25,27], with 1 Hz as the most effective frequency[27].

Thus, it is also useful as a positive control to evaluate the efficacy and efficiency of other neuromodulation policies (see for example[25]). As mentioned above, electrical pulses delivered directly through the MEA (*i.e.*, without the need of an external stimulating electrode) can evoke population responses in rodent brain tissue (see also[28]). Sufficient preservation of neuronal network connectivity within the brain slice, accurate positioning of the brain slice onto the MEA, and the appropriate choice of stimulating electrode pairs allow pursuing electrical neuromodulation experiments that effectively control ictogenesis. As shown in **Figure 5A**, it is possible to reproduce the canonical 1 Hz periodic pacing protocol using planar MEAs both as a recording and as a stimulating device, with the added benefit that the effect of electrical stimulation can be visualized throughout the brain tissue section with a higher number of equally spaced observation points as compared to the conventional field potential recording. **Figure 5B** shows the quantification of the results obtained for n = 9 brain slices, indicating a significant reduction of the total seizing time (total ictal activity duration/observation time[25]) during stimulation (one way-ANOVA, F(df): 6.84(2), $p < 0.01$, Fisher's LSD *post hoc* test, protected). The results are consistent with the literature for external stimulating electrodes[24,25].

To compute the total seizing time, the observation time depends on the interval between ictal events, and it also determines the minimum duration of the stimulation protocol required to confidently evaluate the effects of neuromodulation. We find that recording at least 4 ictal discharges (and hence at least 3 intervals between ictal events) is a good trade-off between collected samples and required collection time. In the control condition (*i.e.*, no stimulation), the observation time is the lag between the onset of the first measured ictal event and the termination of the last one. During neuromodulation, the observation time equals the stimulus protocol duration, since the objective of the measurement is to quantify the phenomena occurring throughout the time that the perturbation is introduced in the system. Computing and comparing the total seizing time rather than the duration and interval of ictal events is the most appropriate parameter to avoid type II error. In fact, along with complete suppression of ictal activity, it is also possible that only one ictal event is generated during electrical stimulation as opposed to the several events observed in the control condition. In this case, whereas measuring the interval between events is not possible, measuring ictal duration as an estimator of stimulation efficacy does not represent the actual outcome of neuromodulation (*i.e.*, ictal activity reduction).

Overall, the results presented here indicate that mouse brain slices coupled to MEAs are valuable tools for epilepsy research and to perform reliable neuromodulation studies that are relevant to advance the field of DBS for epilepsy treatment. In addition, the protocol described here improves brain slice viability and allows pursuing experimental sessions for several hours (**Figure 6**), which may be required, for example, in order to compare the effects of different electrical stimulation paradigms.

FIGURE AND TABLE LEGENDS:

Figure 1: Custom equipment used for this protocol. (**A**) The holding chambers for recovery, pre-warming, and pre-incubation in 4AP are assembled using a beaker and a Petri dish. The Petri dish

should be smaller in diameter than the breaker and is held in place by means of a syringe plunger. The bottom of the Petri dish is replaced by a nylon mesh (from soft stockings) glued with cyanoacrylate onto the rim of the Petri dish. Oxygen is supplied via a bent spinal needle (22 G), inserted between the walls of the Petri dish and the beaker. Bubbles should be emerging from the side of the beaker and never reach the nylon mesh to avoid brain slice displacement. The top of a Petri dish can be used as a lid to avoid ACSF evaporation and maintain oxygen saturation. (**B**) Custom recording chamber. in: inlet reservoir to accommodate the heating cannula and the reference electrode. out: outlet reservoir to accommodate the suction needle. rec: recording chamber. (**C**) Glass Pasteur pipette, curled by fire-polishing, used to handle the tissue and adjust its position within the recording chamber. (**D**) Custom slice hold-down anchor. (**E**) Final assembly of the recording chamber mounted onto the MEA chip. A brain slice rests onto the bottom held in place by the anchor. The red arrow indicates the PTFE tubing covering the heating cannula, whereas the red circle indicates the reference electrode, a saturated KCl pellet submerged by ACSF in the inlet reservoir. The blue arrow indicates the suction needle in the outlet reservoir.

Fig 2: GUI for live MEA electrode mapping. (**A**) Schematic representation of the combined hippocampus-EC slice positioned on the MEA. The default structures used for this protocol are cornu ammonis 3 (CA3), enthorinal cortex (EC), perirhinal cortex (PC), and subiculum (SUB). The reference electrode is schematized as a triangle marker. Electrodes encircled in dotted lines represent the XY coordinates for image straightening. (**B**) Screen capture of the GUI during the live MEA mapping. The flow chart on the left of the capture indicates the step-by-step procedure to follow.

Figure 3: Typical 4AP-induced epileptiform pattern visualized with planar MEAs. (**A**) Mouse brain slice positioned on a planar 6 x 10 MEA (electrode spacing: 500 µm) and side-by-side overview of the 4AP-induced epileptiform pattern visualized with MEA recording. Each square in the grid accommodates the activity recorded at the corresponding location within the brain slice. Dashed blue lines refer to the electrode rows for more clarity. The recording electrode number is identified in blue at the upper-left corner of each square. The grey crossed square represents the reference electrode. (**B**) Representative trace segments of EC and CA3 patterns visualized at a faster time-scale. Arrowheads indicate ictal events. In the EC trace it is possible to appreciate the occurrence of slow interictal discharges (black bar), whereas the CA3 subfield generates the typical sustained fast interictal pattern (black bar). (**C**) Expanded recording of an ictal discharge showing the typical tonic-clonic pattern. (**D**) Fast-scale visualization of the pre-ictal-to-ictal transition corresponding to the EC (red) and CA3 (black) trace segments marked by the red bar in (B). The arrow indicates the onset of the ictal discharge. The superimposed traces highlight that the ictal event originates in the EC and subsequently propagates to the CA3 subfield. (**E**) The expanded view of the interictal periods marked by the black bars in (B) emphasizes the lack of correlation between the slow and fast interictal patterns generated by the EC (red) and CA3 (black), respectively.

Figure 4: Mouse brain slices offer a higher experimental output than rat brain slices. (**A**) Brain slice from a rat (left) and a mouse (right) of matched ages. The rat brain slice is much larger than the MEA recording area and its electrical activity cannot be visualized in full. (**B**) Direct visual

comparison of recurrent ictal activity generated by the mouse and rat enthorinal cortex (EC). (**C**) Mouse and rat brain slices generate ictal discharges of similar duration, but the interval between these events is almost 2-fold in rat brain tissue. * $p < 0.05$. (**D**) As compared to rats, mice offer a 2-fold higher yield of brain slices viable for electrical stimulation experiments aimed at controlling ictogenesis. Electrical stimulation of the subiculum could evoke population responses in the parahippocampal cortices in only 20 of 58 (34%) rat brain slices, as opposed to 33 of 52 (63%) mouse brain slices (Chi^2: 9.22; $p = 0.002$). * $p < 0.05$. (**E**) Among viable slices, the success rate in controlling ictal activity is 2-fold with mouse versus rat brain slices (mouse: 20 of 33 brain slices, 60%: rat: 6 of 20 brain slices, 30%; Chi^2: 4.67; $p = 0.03$). * $p < 0.05$. (**F**) Mouse brain slices can withstand repeated prolonged epochs of sustained electrical stimulation (grey-shaded areas). Upon stimulus withdrawal, mouse brain tissue exhibits full recovery of the epileptiform pattern, whereas rat brain tissue viability falls dramatically at 3 h.

Figure 5: Representative experiment of electrical modulation of ictogenesis using brain slices coupled to MEAs. (**A**) Recordings of 4AP-induced ictal activity in the EC and PC during the control condition (no stimulation), periodic pacing (PP) at 1 Hz (stimulus artifact is truncated), and during recovery upon stimulus withdrawal. (**B**) Quantification of the effect of PP at 1 Hz on the total seizing time. * $p < 0.05$.

Figure 6: Representative long-term recording of 4AP-induced ictal activity. (**A**) Trace segments recorded 8 h after the brain slicing procedure and following 2 h of 4AP application. (**B**) Expanded trace segments corresponding to the boxed ictal discharges identified by the letters **a**, **b**, and **c** in panel (A) show the consistency of generated ictal activity throughout the prolonged observation time-widow.

Table 1: Stock solutions.

Table 2: Solutions composition.

Table 3: Troubleshooting.

DISCUSSION:

MEAs are an invaluable tool for neurophysiology investigations and have reached maturity in recent years thanks to decades of exploration and development. As compared to conventional field potential recording, MEAs offer the great advantage of a higher number of observation points and known inter-electrode distance, which are crucial to accurately pinpoint neuronal network interactions.

The MEA recording technique can also be coupled to other electrophysiology approaches, such as patch-clamp recording[15], to investigate the relation between single neuron and neuronal network activity. Moreover, the possibility of visualizing field potentials and multi-unit activity simultaneously can provide precious insights into the correlation between the activity of small neuronal ensembles and collective neuronal networks. The combination with voltage-sensitive dyes, calcium imaging, and optogenetics[29] allows inferring neurophysiology phenomena using

polyhedral approaches. In addition to pharmacological studies, the possibility of performing both recording and stimulation with the same system makes the MEA recording technique very powerful and versatile: for example, it is possible to study the synaptic plasticity phenomena at the core of memory formation[30], using the neuromodulation protocols presented here, which are relevant to DBS to treat epilepsy and a wide variety of brain disorders. The recent proof that the MEA recording technique can be successfully applied to human epileptic brain tissue[16] demonstrates its invaluable usefulness in epilepsy research, both to understand the basic mechanisms underlying this devastating disease and to fine-tune DBS algorithms to ameliorate it.

However, MEAs force the pursuit of electrophysiology experiments under 'limit' conditions for brain slices, due to the requirement of a submerged recording chamber and the necessity of letting the brain slice rest on the solid substrate where the micro-electrodes are integrated. Thus, brain tissue may not receive adequate oxygen supply, which in turn may affect the quality of the recordings.

The protocol described here allows to reliably record and study ictogenesis in rodent limbic networks coupled to MEAs using the acute *in vitro* 4AP model and to pursue prolonged epochs of electrical stimulation, which provide relevant information for the evaluation of DBS policies. Previous studies on electrical neuromodulation of epileptic limbic networks based on the use of conventional field potential recording have used either mouse or rat brain slices with similar results[24,25]; but the use of rat brain tissue proved more challenging, reasonably due to the weaker connectivity as compared to brain tissue obtained from mice[7]. With regard to the MEA technique, mouse brain slices are best suited by virtue of their smaller size. Moreover, given the higher rate of occurrence of ictal-like discharges in mouse versus rat brain tissue, it is possible to test significantly more brain slices throughout one experimental day, which in turn makes data collection faster and more efficient, and can reduce the number of animals used.

Significance with Respect to Existing Methods:

Ictal discharges recorded using the custom chamber described here appear to be similar in duration and rate of occurrence to those observed using the conventional MEA ring chamber and the same MEA type (planar micro-electrodes, *cf.* [17]). However, it needs to be emphasized that brain slice thickness must be significantly reduced when using the conventional round recording chamber along with a low perfusion rate (1 mL/min), which may hinder the successful pursuit of neuromodulation experiments due to poor connectivity.

In this protocol, a low-volume custom recording chamber inspired by patch-clamp recording chamber design provides stable and reliable laminar flow that is crucial for the successful pursuit of MEA recordings; it also allows increasing the brain slice thickness to 400 µm in order to achieve a fair trade-off between tissue viability and intrinsic connectivity. It is indeed recognized that laminar flow of the recording solution within the chamber is highly desirable for brain slice electrophysiology, since it is not affected by temperature, oxygen, and pH gradients that are observed in circular-flow round recording chambers[19,20,31] (like the ones provided with

commercially available MEA). Such gradients introduce experimental bias and are also detrimental to the brain slice. Recording chambers of a relatively small volume (~1.5 mL) along with a high perfusion rate (5–6 mL/min)[16,31] allow for adequate exchange of the perfusion medium (≥ 3 times/min). The custom chamber can be easily obtained from commercial sources at an affordable price or produced in-house using 3D printing technology. Other studies have reported MEA recordings from 400 µm-thick human brain slices[16] using the conventional MEA ring chamber, while keeping the ACSF volume to 1 mL and enforcing a high perfusion rate (5–6 mL/min) with the help of a low-noise peristaltic pump. However, the authors have used a different model of ictogenesis, *i.e.*, the low Mg^{2+}, which is likely less influenced than the 4AP model by ephaptic mechanisms involving significant increases in extracellular K^+ concentration[11,22]. We have found that a high perfusion rate is not desirable in the 4AP model, possibly due to the fast wash out of accumulated extracellular K^+, which may need to be significantly increased in the recording ACSF[16]. In fact, ictal events appeared more 'chunked' when ASCF perfusion speed was increased to 2–3 mL/min and the brain slice was submerged by a thick ACSF layer, whereas full-blown discharges exhibiting robust tonic-clonic components could be restored upon the return to a lower perfusion rate (1 mL/min) and a thinner ACSF layer right at the tissue surface level (data not shown). The custom recording chamber described in this protocol allows exchanging the perfusion medium 3–5 times/min at a flow rate of 1 mL/min. Thus, the overall oxygen supply to the brain slices is strongly improved even at relatively low perfusion rates, while still guaranteeing a stable recording temperature and high signal-to-noise ratio. Most importantly, it is possible to keep the extracellular K^+ concentration to a physiological value.

The 4AP model of ictogenesis does not require any significant modification of the ACSF ionic composition, such as lowering Mg^{2+} or increasing K^+, and offers the unique advantage of keeping both excitatory and inhibitory transmissions intact[12], an aspect that is highly relevant in epilepsy research in light of the crucial role of both glutamatergic and GABAergic networks in epileptiform synchronization (*cf.* [7]). The 4AP-ACSF used in this protocol contains a physiological K^+ concentration (3.25 mM) and a slightly lower Mg^{2+} concentration than the holding ACSF (1 mM versus 1.3 mM). This concentration still falls within the reported physiological values of Mg^{2+} concentration in the rodent CSF and it is used in many laboratories (see for example[17,18]). To the purpose of the described protocol, we have found that this slight decrease is preferred to aid in the effect of 4AP.

In previous work, 4AP has been applied to the brain slice when it was already positioned inside the recording chamber[17,18]. Unless the experimenter needs to observe the time latency between the 4AP application and the onset of epileptiform patterns, we find that this approach is rather time-consuming and is not suitable to pursue the prolonged recording and stimulation sessions described in this protocol. Pre-incubation of brain slices in 4AP at 32 °C allows sparing much experimental time, since brain slices can be pre-treated in series while pursuing the experiment using other tissue sections.

The prolonged viability of brain slices may be useful to analyze network features in the long-term. Moreover, their improved resilience to repetitive electrical stimulation is of great advantage if

the experimenter wants to compare several stimulation protocols, which must be performed in the same brain slice for statistical robustness. In our hands, when testing 3 stimulation protocols, each preceded by a control phase and followed by a recovery phase, the experiment may last 3–5 h. In this context, live MEA mapping is crucial in order to activate the correct pathway and suppress rather than favor ictogenesis via electrical stimulation. Our user-friendly GUI represents a quick, simple and flexible tool to map the electrodes of different brain structures. Contrary to commercial software, it is possible to add custom MEA layouts even with basic programming skills. Images can be acquired in bright field; thus, any general purpose camera that has good resolution and fits on a microscope is suitable. An inverted microscope is essential to visualize the microelectrodes position relative to the brain slice, since these would be hidden underneath the tissue if using an upright microscope. However, a stereo-microscope is recommended in addition to the inverted type if the experimenter needs to perform knife-cuts to disrupt specific neuronal pathways.

Finally, a further advantage of the proposed approach is the cheap and relatively easy assembly of most of the required tools, like the custom recording chamber and the holding chambers, the warm bath and the slice anchor, as well as the unnecessary use of a costly low-noise peristaltic pump.

Limitations of the Technique:

MEA recording does not allow visualizing very slow waves, *i.e.*, DC shifts in the signal. Such baseline deflections may help asymptotic measurement of ictal discharge duration and, most important, they are fundamental to study cortical spreading depression (a phenomenon that relates to sudden unexpected death in epilepsy[32] and is shared between epilepsy and migraine[33]). With regard to electrical neuromodulation, the protocol described here allows performing several stimulation sessions without affecting brain slice viability. We could successfully perform up to 3 stimulation sessions of 20–45 min, similar to what reported in previous work[25]. Although brain slices may probably withstand a higher number of stimulation sessions or longer stimulation protocols, we did not test brain slices in this regard. We recommend limiting the number of stimulation protocols to 3 and avoiding prolonged (≥ 60 min) stimulation sessions, which may significantly stress brain tissue maintained under these limit conditions, up to the lack of recovery upon stimulus withdrawal.

Critical Steps Within the Protocol:

Several critical factors may hinder the successful pursuit of recording and modulation of the epileptiform activity in brain slices coupled to MEA. Besides the rodent species used and the quality of the brain slices, the perfusion rate during recording and the dynamics of the ACSF flow (*i.e.*, laminar versus circular) within the recording chamber, we have found that the conditions of recovery and long-term maintenance of brain slices as well as the mode of 4AP application are the most critical steps.

When using a submerged holding chamber it is crucial to store the brain slices at room

temperature to preserve brain tissue network activity and favor the induction of ictal-like discharges by 4AP application. In our hands, recovery and maintenance at 32 °C deteriorated the brain tissue within 3–4 h of slicing.

Incubation of brain slices in 4AP-ACSF at room temperature and recording at 32 °C is, however, not desirable. We have found many difficulties in observing robust recurrent ictal discharges in this condition. Indeed, low temperatures in the 20–24 °C range have been reported to dampen or even prevent 4AP-induced ictogenesis both *in vitro*[34] and *in vivo*[35]. Thus, the 4AP incubation and recording temperatures must fall within the 30–34 °C and must be matched. To avoid putting the brain tissue under too much stress due to the simultaneous induction of hyperexcitability by 4AP and the sudden exposure of brain slices from room temperature to the warm (32 °C) 4AP-ACSF, it is fundamental to perform an intermediate pre-warming step and let the brain slices habituate in the holding ACSF at 32 °C for 20–30 min. Skipping this step may affect ictogenesis induction by 4AP.

Another aspect that needs to be mentioned is the importance of D-glucose concentration in the ACSF after slicing. A 25 mM concentration preserves the brain slices better than the 10 mM concentration used in many laboratories and it also improves the rate of occurrence of ictal activity, which is 2-fold faster (thus, making it easier to pursue a long series of experimental protocols in several brain slices each day).

Lastly, some important fine details during the slicing procedure need to be mentioned. First, do not allow the the intact brain and the brain slices to freeze in the cold cutting ACSF. Chilling the brain for < 2 min is sufficient given the small size of the mouse brain. Tissue sections should be transferred immediately from the buffer tray to the rinsing beakers at room temperature. Rinsing the cutting ACSF is very important otherwise the high sucrose concentration will make the brain slices stick to the nylon mesh of the holding chamber. To effectively rinse the tissue, it is important to minimize the cutting ACSF content within the transfer pipette so as to not contaminate the holding ACSF excessively. The 2-stage rinsing aids in minimizing contamination. Upon completion of the slicing procedure, tissue sections should be transferred immediately from the rinsing beakers to the holding chamber, where the soft nylon mesh suspended half-way in the holding ACSF allows tissue oxygenation on both sides. The same principle should be applied at all stages following the slicing procedure: brain slices should never sit at the bottom of the beaker, they should not be in contact with the sides of the holding chambers, and they should never be in contact with each other nor overlap.

Modifications and Troubleshooting:

ACSF composition may be modified according to the experimenter's needs. For example, drugs may be added to dissect the contribution of specific neurotransmitters or ion channels to the efficacy of electrical neuromodulation. Moreover, pyruvic and ascorbic acid (*cf.* **Table 2**) may be omitted, although we find that they exert a powerful neuroprotective role. We report in **Table 3** the most common issues that can be encountered in this protocol and how to deal with them.

Conclusions:

MEA recording is undoubtedly an invaluable technique to address the interactions of neuronal networks in health and disease. In addition to pharmacological studies, it is also possible to evaluate electrical neuromodulation protocols that are relevant to DBS applied to epilepsy and other neurological disorders. In this protocol, we have shown that it is possible to reproduce typical periodic stimulation experiments with similar results to those obtained with conventional extracellular field potential recording and external stimulating electrodes. The increasing availability of commercial user-friendly equipment and advanced software tools make the MEA recording technique also suitable for closed-loop stimulation experiments, to provide *ad hoc* stimulation to the brain tissue, and to investigate the contribution of feedback mechanisms to neuronal network responses.

ACKNOWLEDGMENTS:

G.P. is a Marie Skłodowska-Curie Fellow funded by the European Union MSCA-IF-2014 project Re.B.Us, G.A. n.660689, under the framework program H2020. The GUI *mapMEA* is freely available upon request to author ilaria.colombi@iit.it.

DISCLOSURES:

The authors have nothing to disclose.

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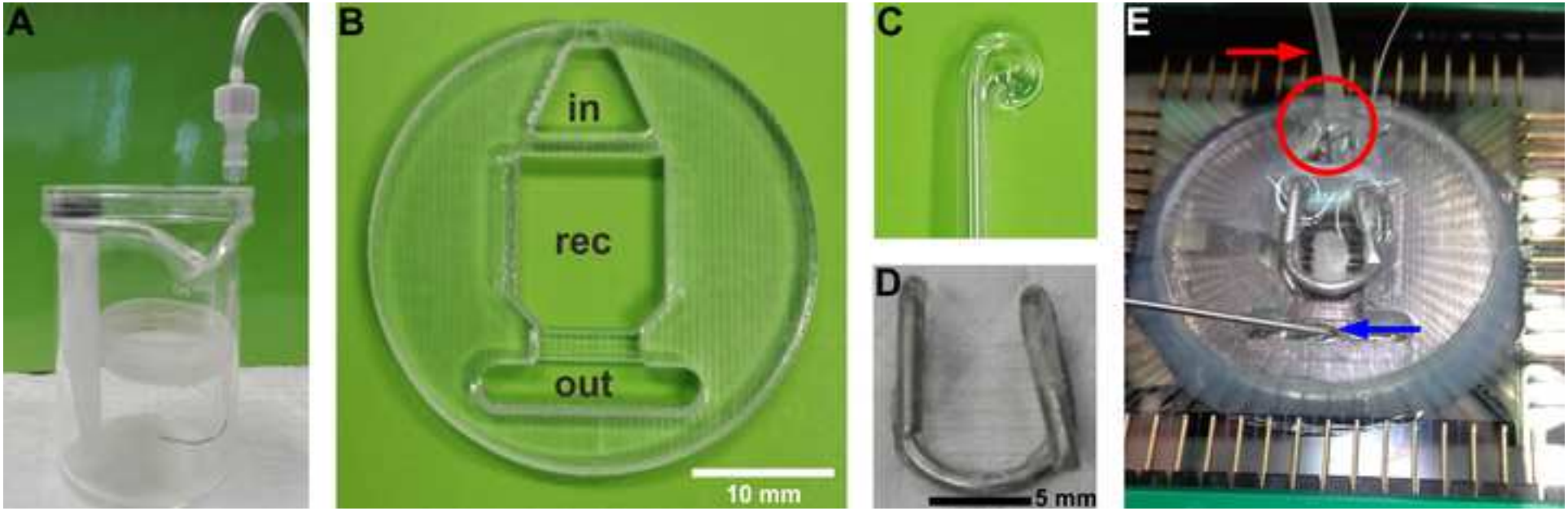
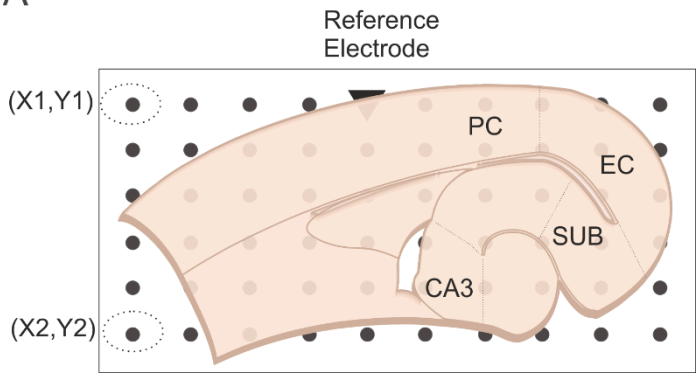


Figure 2

A



B

1) Press «**Browse button**» and load picture

2) Press «**Activate pointer button**»

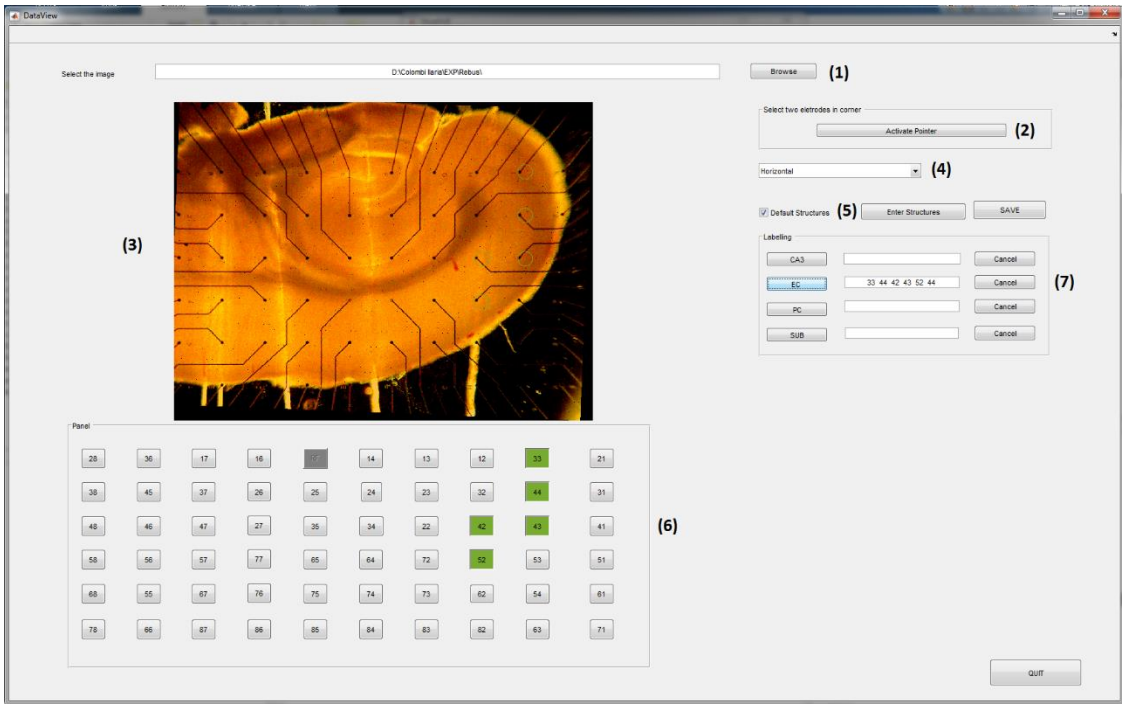
3) Select (X1,Y1) and (X2,Y2) in the picture

4) Drop-down menu select **'Horizontal'**

5) Check «**Default structures**»

6) Press the pushbuttons of electrodes

7) Click pushbutton in the structures panel



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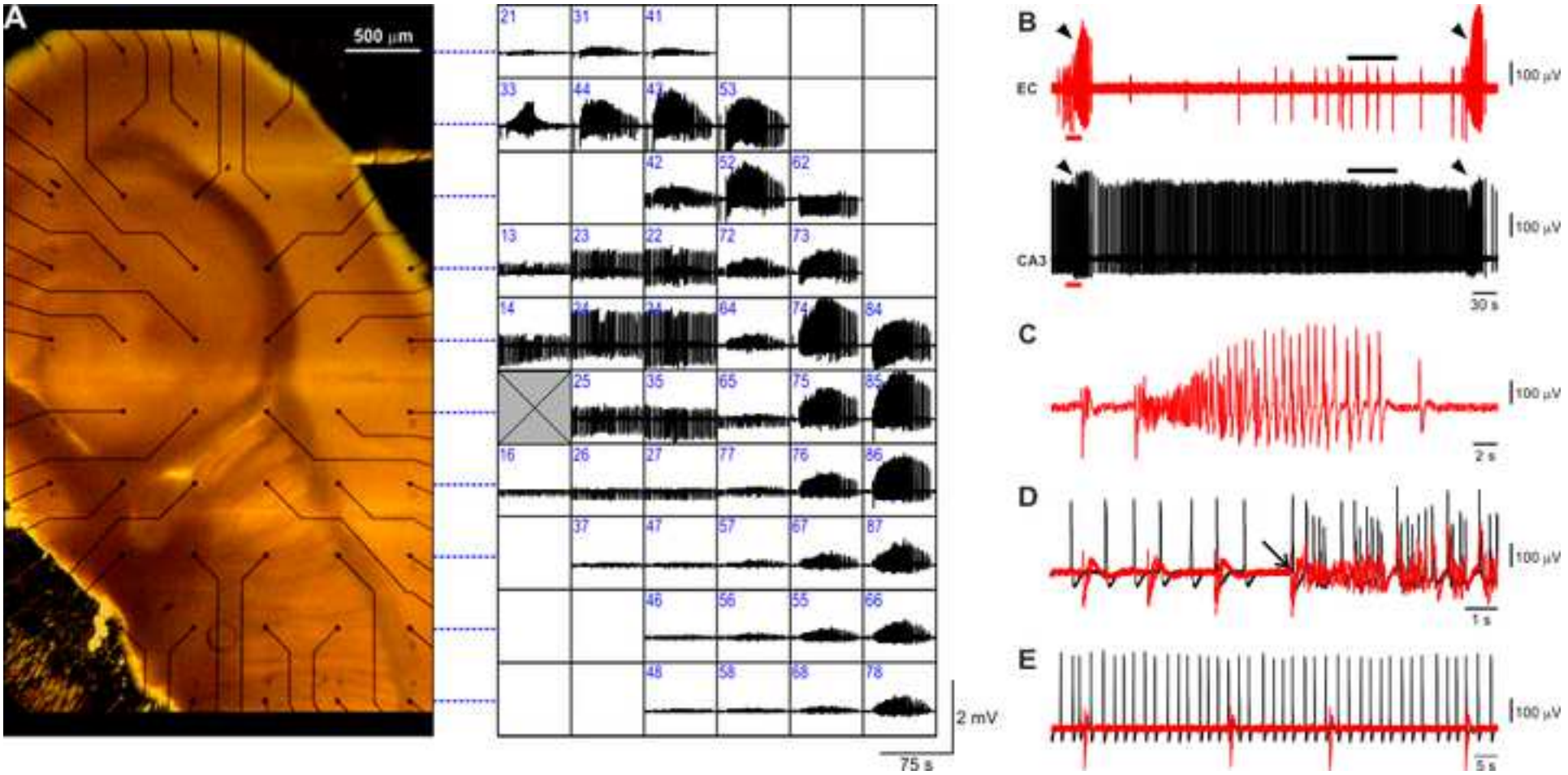

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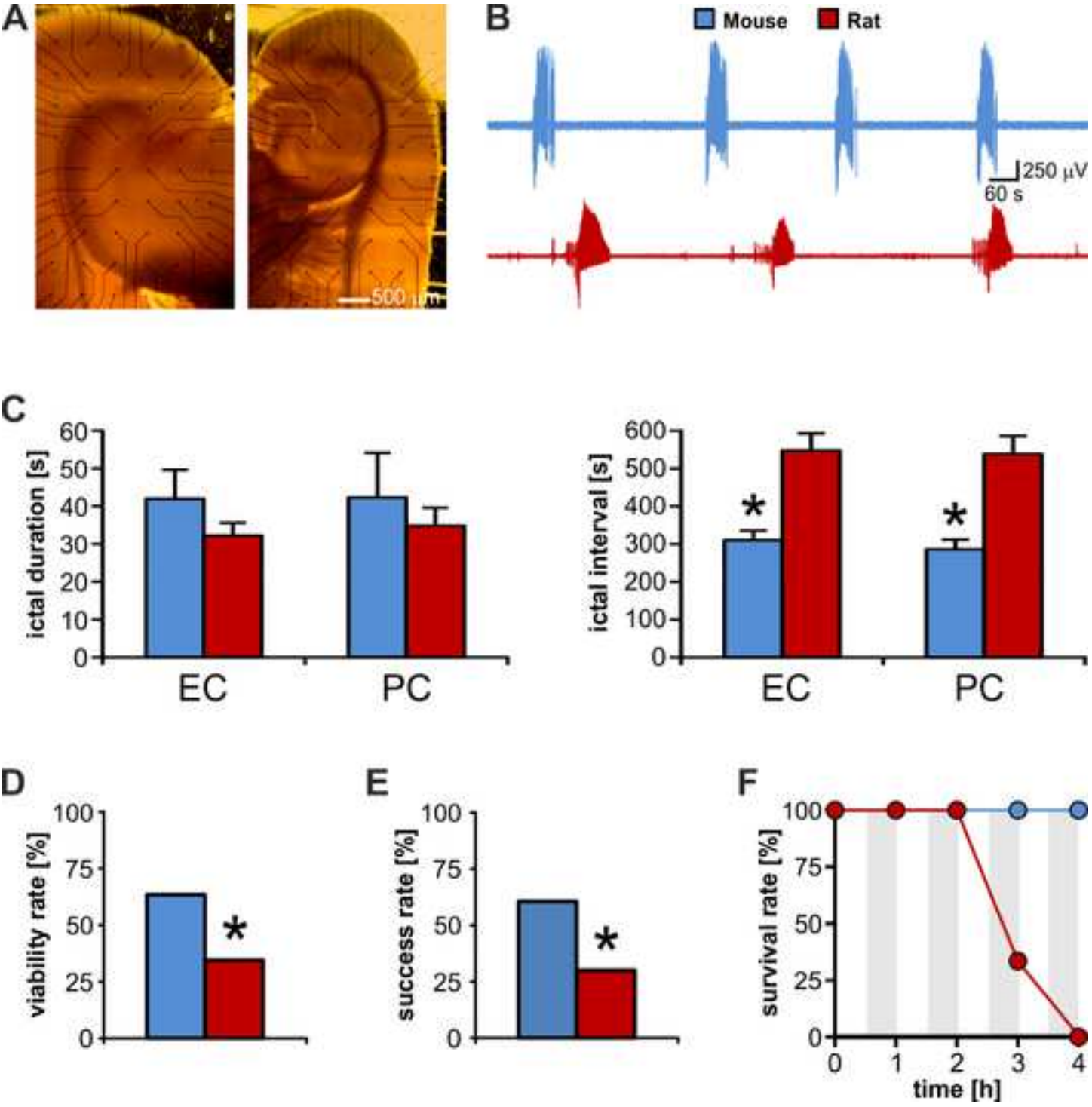

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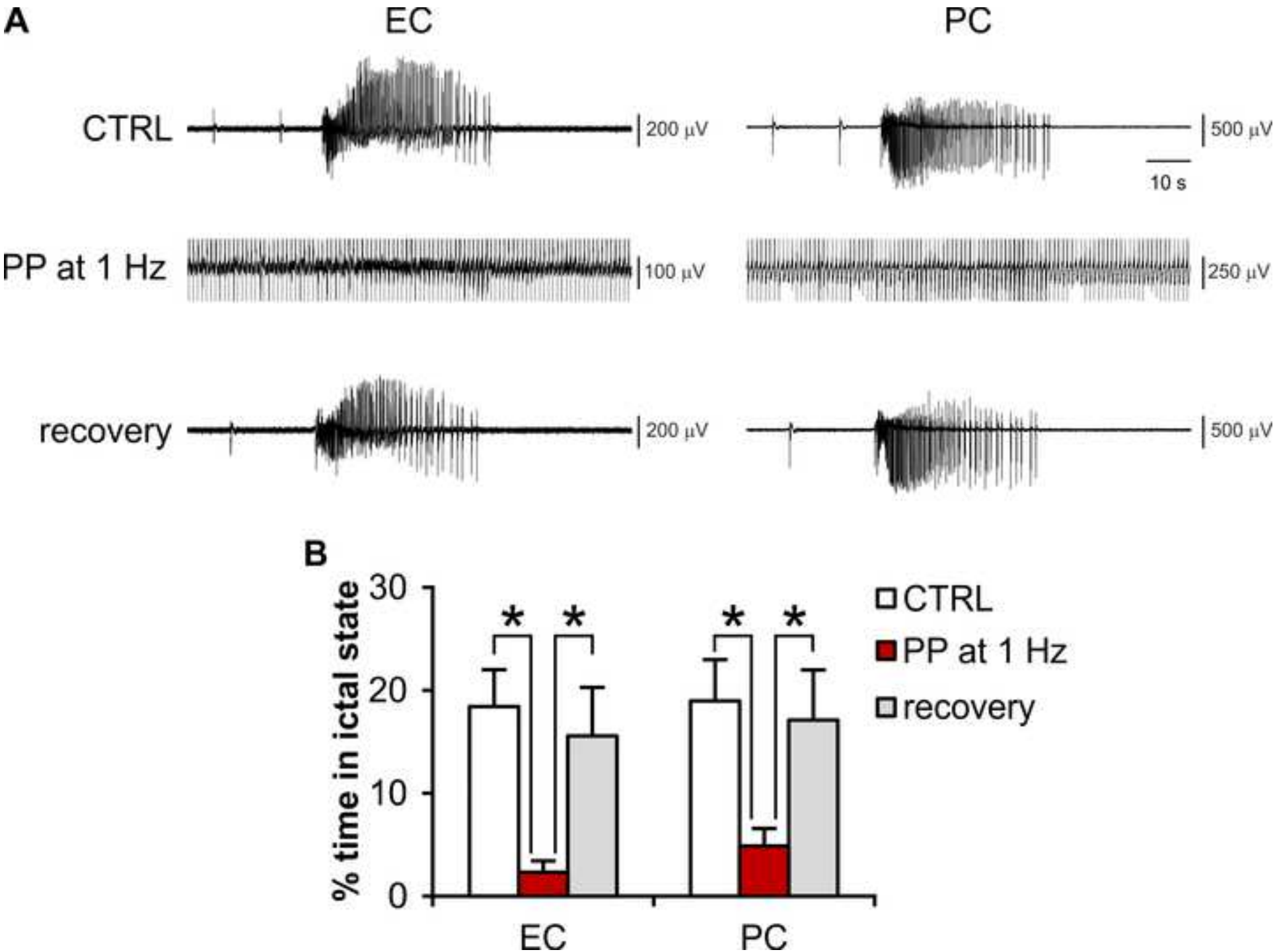

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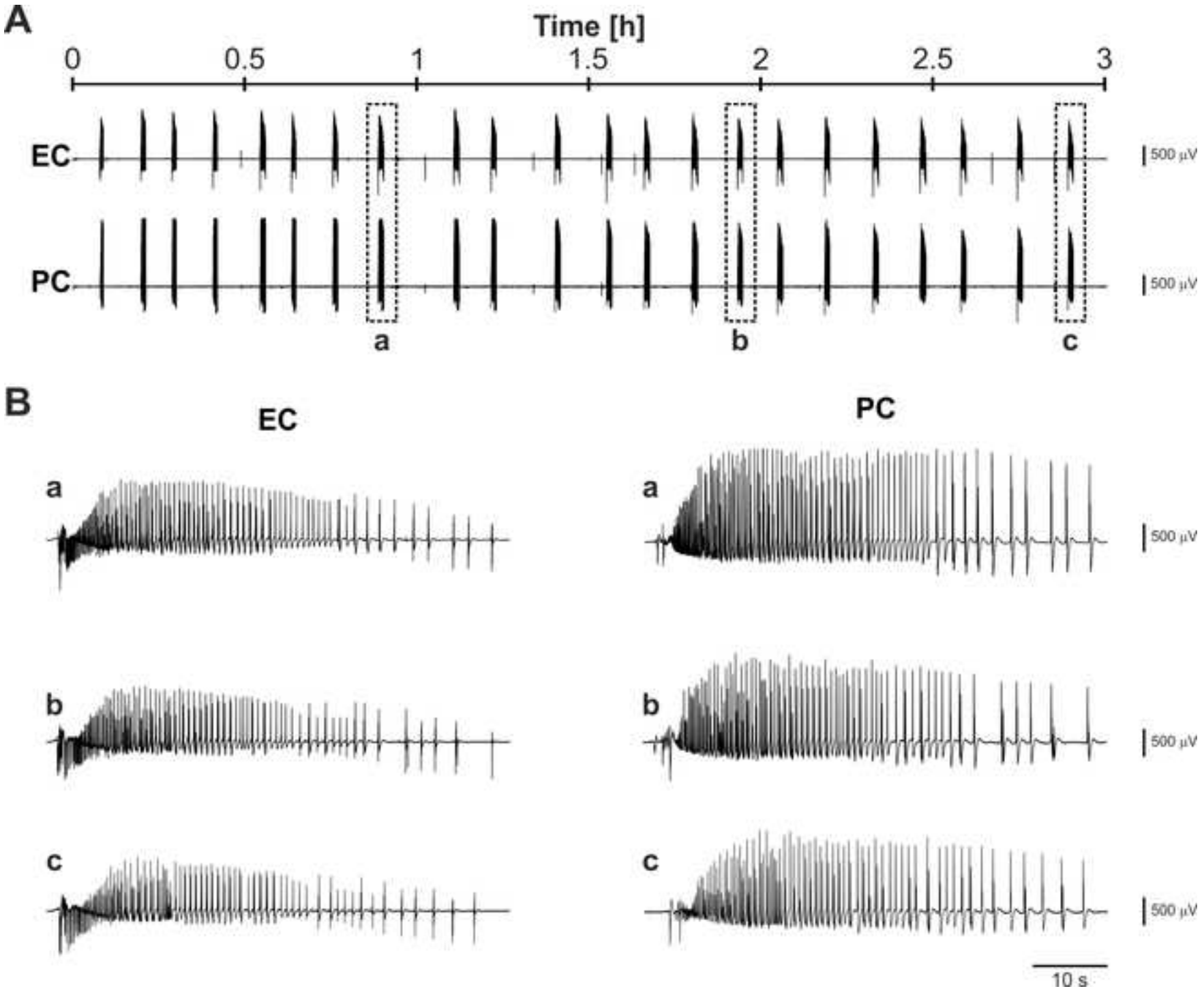

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Stock A

Solvent: **distilled water**
Concentrate: **10X**
Storage temperature: **4 °C**
Maximum storage time: **1 wk**
note: filter using a 50 μm membrane filter

Chemical	Molecular weight	Concentration (mM)
NaCl	58.44	1150
KCl	74.55	20
KH_2PO_4	136.1	12.5
$CaCl_2$*2 H_2O	147.02	20
D-Glucose	180.2	250

Stock B

Solvent: **distilled water**
Concentrate: **10X**
Storage temperature: **4 °C**
Maximum storage time: **1 wk**
note: filter using a 50 μm membrane filter

Chemical	Molecular weight	Concentration (mM)
$NaHCO_3$	84.01	260

Stock C

Solvent: **distilled water**
Concentrate: **10X**
Storage temperature: **4 °C**
Maximum storage time: **2 wks**
note: filter using a 50 μm membrane filter

Chemical	Molecular weight	Concentration (mM)
KCl	74.55	20
KH_2PO_4	136.1	12.5
$MgCl_2$*6 H_2O	203.3	50
$MgSO_4$*6 H_2O	246.47	20
$CaCl_2$*2 H_2O	147.02	5

Stock M

Solvent: **distilled water**
Concentrate: **100X**
Storage temperature: **4 °C**
Maximum storage time: **4 wks**

Chemical	Molecular weight	Concentration (mM)
$MgSO_4$*6 H_2O	246.47	100

4AP stock

Solvent: **distilled water**
Concentrate: **1000X**
Storage temperature: **4 °C**
Maximum storage time: **2 wks**
note: vortex for 2-3 min or stirr for 30-60 min

Chemical	Molecular weight	Concentration (mM)
4-aminopyridine	94.11	250

Note: CAUTION! 4-AP is toxic and cinvulsant. Use gloves and avoid spreading or spilling.

Table 2

Click here to download Table Table 2.xlsx

HOLDING ACSF	
Chemical	**C [mM]**
NaCl	115
KCl	2
KH_2PO4	1.25
$MgSO_4$	1.3
$CaCl_2$	2
D-glucose	25
$NaHCO_3$	26
L-Ascorbic Acid	1
pH	7.4
Osmolality	300 mOsm/Kg

RECORDING ACSF	
Chemical	**C [mM]**
NaCl	115
KCl	2
KH_2PO4	1.25
$MgSO_4$	1
$CaCl_2$	2
D-glucose	25
$NaHCO_3$	26
L-Ascorbic Acid	1
pH	7.4
Osmolality	300 mOsm/Kg

CUTTIN(	
Chemical	
Sucrose	
KCl	
KH_2PO4	
$MgCl_2$	
$MgSO_4$	
$CaCl_2$	
D-glucose	
$NaHCO_3$	
L-Ascorbic Acid	
Pyruvic Acid	
pH	
Osmolality	

ɜ ACSF

C [mM]
208
2
1.25
5
2
0.5
10
26
1
3
7.4
300 mOsm/Kg

Table 3 Click here to download Table Table 3.xlsx

Troubleshooting	
ISSUE	
Ictal discharges look 'chunked'	Decrease the perfusio brain slice by adjustin
No ictal activity	**(1)** Verify that CA3-dri cortex. Use a small sca Schaffer Collaterals if of the slice hold-down suited, whereas the in **(2)** Check the slice viab activity? Wait until abo ictal activity does not
The brain slice moves when placing the hold-down anchor	**(1)** Gently remove the **(2)** Check that the slice **(3)** Remove the 4AP-A slice adhesion to the M **(4)** Reposition the slice
The brain slice floats on the MEA chip after being transferred	**(1)** Gently aspirate exc **(2)** The MEA chip migh
Electrical stimulation does not elicit network responses	Increase stimulus inte
Electrical stimulation can elicit population responses in the proximal but not distal cortical areas	The issue is ikely due t Electrical stimulation r only. It is recommende
Signal is noisy	**(1)** Check the referenc by the incomplete fill electrodes does not cc **(2)** Check the overall g **(3)** Adjust the suction suction sound. Grounc **(4)** Clean the MEA exte **(5)** The MEA chip may and the signal-to-noise

COUNTERMEASURES
n rate and/or lower the ACSF volume above the g the suction needle position.
ven fast interictal events do not propagate to the ılpel blade (e.g., n.10) or a needle to sever the need be, but be careful not to cut the nylon threads anchor. A stereo- or upright microscope are best verted microscope makes this task very difficult. ɔility: can strong electrical stimulation trigger ictal ɔut 2 h of 4AP application, then change brain slice if ɔccur.
slice anchor and reposition the brain slice. e anchor is evenly wet of 4AP-ACSF. CSF from the recording chamber to favor the brain /IEA. e hold-down anchor.
:ess ACSF with a Pasteur pipette. ıt need to be re-coated.
nsity, change electrode pairs.
:o poor connectivity or too low stimulus intensity. may be ineffective or effective in some cortical areas ed to change brain slice.
:e electrode: noisy baseline can at times be caused of the inlet reservoir such that the reference ɔmpletely deep into the ACSF. ;rounding of equipment. needle position so as to hear a constant, soft d the suction needle. ernal contacts with ethanol using a cotton swab. be damaged: change MEA chip and check artifacts e ratio.

Materials Table

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Name of Material/ Equipment	Company	Catalog Number
Poly-D-Lysine	Sigma-Adrich	P7886
NaCl	Sigma-Adrich	S9888
KCl	Sigma-Adrich	P9541
KH_2PO_4	Sigma-Adrich	795488
$CaCl_2$*2 H_2O	Sigma-Adrich	C3306
D-Glucose	Sigma-Adrich	RDD016
$NaHCO_3$	Sigma-Adrich	S5761
$MgCl_2$*6 H_2O	Sigma-Adrich	M2670
$MgSO_4$*6 H_2O	Sigma-Adrich	M5921
Sucrose	Sigma-Adrich	RDD023
Pyruvic Acid, 98%	Sigma-Adrich	107360
4-aminopyridine	Sigma-Adrich	A78403
STG-2004	Multichannel System	STG4004-1.6mA
MEA1060	Multichannel System	N/A
planar MEA	Multichannel System	60MEA500/30iR-Ti
McRack	Multichannel System	
McStimulus II	Multichannel System	
TC02	Multichannel System	TC02
PH01	Multichannel System	PH01
MPH	Multichannel System	MPH
Elastostil E43	Wacker	E43
MEA Custom Chamber	Crisel Instrument	SKE-chamber MEA
Ag/AgCl electrode, pellet, 1.0 mm	Crisel Instrument	64-1309
Tergazyme	Sigma-Adrich	Z273287-1EA
MATLAB	The Mathworks	
VT1000S	Leica Biosystems	VT1000S
	Warner Instruments	64-1309

Comments/Description
Needed for MEA coating
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Chemical
Convulsant drug
4-channel stimulus generator with voltage (±8 V) and current output (±1.6 mA)
MEA amplifier
Must be without ring to allow using the custom recordingchamber
Recording software
STG4004 control software
2-channel thermostat
Heating perfusion canula
Magnetic holder for PH01 and suction needle
Elastomeric sealant used to mount the custom recording chamber onto the MEA
Custom recording chamber
Reference electrode for the custom recording chamber
Enzymatic cleaner
Programming environment for electrodemapping
Vibratome
Ag-AgCl Electrode Pellet 1.0 mm (E205). Reference electrode.

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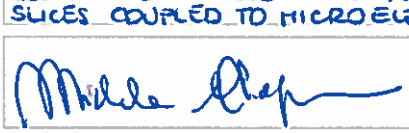
Signature:	
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We have carefully read the manuscript and we have used the spell-check feature of our word processor software. The manuscript should now be free of errors/typos.

2. **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.

3. **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.

4. **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.

5. **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.

6. **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.

7. **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.

8. **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.

9. **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.

10. **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.

11. **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.

12. **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.

2. **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.

3. **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.

4. **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.

5. **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 Boido et 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.

6. **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.

7. **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.

8. **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.

9. **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.

10. **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)

11. **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.

2. **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.

3. **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.

4. **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.

5. **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.

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

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

7. **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?

8. **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.

9. **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.

10. **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.

2. **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.

3. **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.

4. **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.

5. **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.

2. **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.

3. **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.

4. **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.

5. **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.

6. **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).

7. **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.

8. **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.

9. **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.

10. **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**.

11. **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.

12. **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.

2. **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.

3. **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.

4. **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.