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Acute Mouse Brain Slicing to Investigate Spontaneous Hippocampal Network Activity

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Acute brain slicing has historically been one of the most powerful experimental methods available to neuroscientists, and over the past several decades numerous modifications have been introduced to suite particular objectives. In order to gain meaningful insight into the function and organization of neural circuits through *in vitro* experimentation, it is essential for researchers to prepare brain tissue samples in a way that optimizes the integrity and health of the tissue. Suboptimal preparation of tissue sections can result in poor quality data and make it difficult to draw meaningful conclusions from electrophysiological recordings. This limitation is particularly evident in studies of network-level neural activity in isolated brain sections, which require that a large proportion of cells within the tissue section be healthy and spontaneously active.

The protocol retitled *An acute mouse brain slicing method for investigation of spontaneous hippocampal network activity* is an appropriate submission for the Journal of Visualized Experiments. The steps described in this protocol require numerous experimental techniques that are best demonstrated visually, and as such JoVE offers a unique and valuable avenue for this procedure to be demonstrated and disseminated to a wide audience. It is my hope that publication of this manuscript and the associated video (to be produced at a later date by JoVE) will allow researchers to learn the necessary techniques to create healthy acute brain slices suitable for a wide range of experiments.

Sincerely,

Alex C. Whitebirch

TITLE:

Acute Mouse Brain Slicing to Investigate Spontaneous Hippocampal Network Activity

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KEYWORDS:

hippocampus, mouse, slice, electrophysiology, sharp-wave ripple, spontaneous activity, hippocampal-entorhinal cortex network, interface chamber

SUMMARY:

This protocol describes the preparation of horizontal hippocampal-entorhinal cortex (HEC) slices from mice exhibiting spontaneous sharp-wave ripple activity. Slices are incubated in a simplified interface holding chamber and recordings are performed under submerged conditions with fast-flowing artificial cerebrospinal fluid to promote tissue oxygenation and the spontaneous emergence of network-level activity.

ABSTRACT:

Acute rodent brain slicing offers a tractable experimental approach to gain insight into the organization and function of neural circuits with single-cell resolution using electrophysiology, microscopy, and pharmacology. However, a major consideration in the design of in vitro experiments is the extent to which different slice preparations recapitulate naturalistic patterns of neural activity as observed in vivo. In the intact brain, the hippocampal network generates highly synchronized population activity reflective of the behavioral state of the animal, as exemplified by the sharp-wave ripple complexes (SWRs) that occur during waking consummatory states or non-REM sleep. SWRs and other forms of spontaneous network activity can emerge spontaneously in isolated hippocampal slices under appropriate conditions. In order to apply the powerful brain slice toolkit to the investigation of hippocampal network activity, it is necessary to utilize an approach that optimizes tissue health and the preservation of functional connectivity within the hippocampal network. Mice are transcardially perfused with cold sucrose-based artificial cerebrospinal fluid. Horizontal slices containing the hippocampus are cut at a thickness of 450 μm to preserve synaptic connectivity. Slices recover in an interface-style chamber and are transferred to a submerged chamber for recordings. The recording chamber is designed for dual surface superfusion of artificial cerebrospinal fluid at a high flow rate to improve oxygenation of the slice. This protocol yields healthy tissue suitable for the investigation of complex and spontaneous network activity in vitro.

INTRODUCTION:

Electrophysiological measurement from living hippocampal slices in vitro is a powerful experimental approach with numerous advantages. The experimenter can use a microscope, micromanipulators, and a recording system to directly visualize and collect measurements from individual neurons in the tissue. Tissue slices are also very accessible to photostimulation or drug delivery for optogenetic, chemogenetic, or pharmacological experiments.

The hippocampal network generates highly synchronous population activity in vivo, visible as oscillations in the extracellular local field potential[1-5]. Brain slice methods have been leveraged to gain insight into the cellular and circuit mechanisms underlying these neuronal network oscillations. Foundational work from Maier et al. demonstrated that sharp wave-ripple complexes (SWRs) can emerge spontaneously in slices of the ventral hippocampus[6,7]. Subsequent studies from multiple investigators have gradually elucidated many aspects of SWRs, including the role of neuromodulators in regulating the network state of the hippocampus[8-10] and the synaptic mechanisms that drive the in vitro reactivation of neuronal ensembles previously active during behavior in vivo[11]. Brain slice experiments have also provided insight into the gamma range oscillation (30–100 Hz), a distinct hippocampal network state believed to support memory encoding and recall[12,13]. Finally, recognizing the central role of the hippocampus and associated structures in the pathophysiology of temporal lobe epilepsy[14,15], researchers have used hippocampal slice preparations to investigate the generation and propagation of epileptiform activity. Carter et al. demonstrated that combined hippocampal-entorhinal cortex slices prepared from chronically epileptic animals can spontaneously generate epileptiform discharges in vitro[16]. Subsequently, Karlócai et al. explored the mechanisms underlying epileptiform discharges in hippocampal slices by using modified artificial cerebrospinal fluid (ACSF) with altered ion concentrations (reduced Mg^{2+} or elevated K^{+}) or added drugs (4AP or gabazine)[17].

Investigators have developed numerous hippocampal slice approaches that differ in key ways: (1) the region of the hippocampus contained in the slice (dorsal, intermediate, or ventral); (2) the presence or absence of extrahippocampal tissues such as the entorhinal cortex; (3) the orientation used to cut slices (coronal, sagittal, horizontal, or oblique); and (4) the conditions under which the tissue is maintained after slicing (submerged fully in ACSF or held at the interface of ACSF and humidified, carbogen-rich air).

The choice of which slicing approach to use should be determined by the experimental objective. For example, transverse or coronal slices of the dorsal hippocampus maintained under submerged conditions have been used very effectively for the investigation of intrahippocampal circuitry and synaptic plasticity[18-20]. However, such preparations do not spontaneously generate network oscillations as readily as slices from the ventral hippocampus[21-23]. Although a state of persistent SWR activity can be induced by tetanic stimulation in transverse slices from the dorsal and ventral hippocampus[24], spontaneous SWRs are more readily observed in ventral slices[7,25].

An inherent physiological and anatomical distinction between the dorsal and ventral hippocampus is supported by studies performed both in vivo and in vitro[26]. Recordings in rats revealed strongly coherent theta rhythms throughout the dorsal and intermediate hippocampus, yet poor coherence between the ventral region and the rest of the hippocampus[27]. SWRs in vivo

propagate readily between the dorsal and intermediate hippocampus, while SWRs that originate in the ventral hippocampus often remain local[28]. The associational projections originating from CA3 pyramidal neurons that reside in the dorsal and intermediate hippocampus project long distances along the longitudinal axis of the hippocampus. CA3 projections originating from ventral regions remain relatively local, and thus are less likely to be severed during the slicing process[29,30]. Ventral slices may, therefore, better preserve the recurrent network necessary to generate population synchrony. The propensity of ventral slices to generate spontaneous network activities in vitro may also reflect higher intrinsic excitability of pyramidal neurons or weaker GABAergic inhibition in the ventral hippocampus as compared to more dorsal regions[31]. Indeed, ventral hippocampal slices are more susceptible to epileptiform activity[32,33]. Thus, many studies of spontaneous physiological[8,9,11,24] or pathological[16,34-36] network oscillations have traditionally used a horizontal slicing approach, sometimes with a slight angle in the fronto-occipital direction, which yields tissue slices parallel to the transverse plane of the ventral hippocampus.

Network connectivity is unavoidably impacted by the slicing procedure as many cells in the slice will be severed. The angle and thickness of the slice and the tissue retained in the preparation should be considered to optimize connectivity in the circuits of interest. Many studies have utilized horizontal combined hippocampal-entorhinal cortex slices (HEC) to explore interactions between the two structures in the context of physiological or pathological network oscillations. Roth et al. performed dual recordings from the CA1 subfield of the hippocampus and layer V of the medial entorhinal cortex to demonstrate propagation of SWR activity through the HEC slice[37]. Many studies of epileptiform activity have used the HEC slice preparation to investigate how epileptiform discharges propagate through the corticohippocampal network[16,35,36,38]. It is important to note that preservation of the intact corticohippocampal loop is not a prerequisite for spontaneous SWRs, epileptiform discharges, or gamma oscillations; network oscillations can be generated in transverse slices of the dorsal or ventral hippocampus with no attached parahippocampal tissues[21-23, 25,39-41]. A more important factor for the spontaneous generation of network oscillations in hippocampal slices may be the thickness of each slice, as a thicker slice (400–550 μm) will preserve more connectivity in the CA2/CA3 recurrent network[21,22,25].

Although angled horizontal HEC slices (cut with an approximately 12˚ angle in the fronto-occipital direction) have been used to study the functional connectivity of the corticohippocampal loop[11,16,34,35,42], such angled preparations are not required for spontaneous network activity[43-45]. However, the use of an angled slicing plane does allow the investigator to selectively make slices that best preserve the transversely-oriented lamellae of either the ventral or intermediate hippocampus, depending on whether a downward or an upward angle is applied (**Figure 1**). This approach is conceptually similar to that used by Papatheodoropoulos et al., 2002, who dissected each hippocampus free and then used a tissue chopper to create transverse slices along the entire dorsal-ventral axis[21]. In the light of the aforementioned functional distinctions between the ventral and dorsal-intermediate hippocampus, investigators should consider the anatomical origin of slices when designing experiments or interpreting results. Using an agar ramp during the slicing procedure is a simple way to preferentially produce slices from either the intermediate or ventral hippocampus.

Hippocampal slices can be maintained in either a submerged chamber (with the tissue fully immersed in ACSF), or an interface-style chamber (e.g., Oslo or Haas chamber, with slices covered only by a thin film of flowing media). Interface maintenance enhances oxygenation of the tissue, which promotes neuronal survival and allows for sustained high levels of interneuronal activity. Traditionally, submerged recording conditions use a slower ACSF flow rate that does not provide adequate tissue oxygenation for stable expression of network-level oscillations. In submerged hippocampal slices carbachol-induced gamma oscillations are only observed transiently[46,47], while they can be stably maintained in interface recording chambers[10,48,49]. As such, many studies of complex spontaneous activity in vitro have relied on interface recording chambers to investigate sharp-wave ripple complexes[6-10,25,37], gamma oscillations[10,13], and epileptiform activity[16,38,45,47].

In a submerged-style recording chamber, an immersion microscope objective can be used to visualize individual cells and selectively target healthy-looking cells for recordings. The submerged preparation also allows fine control over the cellular milieu, as submersion facilitates rapid diffusion of drugs or other compounds to the tissue. Thus, a modified methodology in which stable network oscillations are maintained under submerged conditions represents a powerful experimental approach. This approach is exemplified by the work of Hájos et al., in which hippocampal slices recover in a simplified interface-style holding chamber for several hours before transfer to a modified submerged recording chamber with a high flow rate of ACSF (~6 mL/min) to enhance oxygen supply to the tissue[12,48,49]. Under these conditions, high levels of interneuron activity and stable spontaneous network oscillations can be maintained in a submerged recording chamber. This modified approach allows the investigators to perform visually guided whole-cell patch clamp recordings and characterize the contribution of morphologically identified cell types to carbachol-induced gamma oscillations[12]. SWRs can also occur spontaneously in submerged hippocampal slices with a fast flow rate of ACSF[11,48,49]. Maier et al. demonstrated that hippocampal slices that recovered in an interface chamber before transfer to a submerged recording chamber reliably exhibited spontaneous SWRs, whereas slices that recovered submerged in a beaker before transfer to a submerged recording chamber showed smaller evoked field responses, lower levels of spontaneous synaptic currents, and only very rarely exhibited spontaneous SWRs[43]. Schlingloff et al. used this improved methodology to demonstrate the role of parvalbumin-expressing basket cells in the generation of spontaneous SWRs[44].

The following protocol presents a slicing method through which spontaneously active neurons in horizontal hippocampal slices can be recovered under interface conditions and subsequently maintained in a submerged recording chamber suitable for pharmacological or optogenetic manipulations and visually guided recordings.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee at Columbia University (AC-AAAU9451).

1. Prepare solutions

1.1. Prepare sucrose cutting solution for slicing as described in **Table 1**.

NOTE: After preparing 1 L of sucrose solution, freeze a small amount (approximately 100–200 mL) in an ice tray. These frozen sucrose ice cubes will be blended into an icy slurry (see step 4.3).

1.2. Prepare artificial cerebrospinal fluid (ACSF) for recording as described in **Table 2**.

1.3. Prepare 1 M of NaCl for stimulation pipettes by dissolving 0.05844 g of NaCl in 1 mL of purified water that has been filtered to remove trace metals and other impurities.

NOTE: Sucrose cutting solution and ACSF should be prepared fresh for each experiment.

2. Prepare agar ramp

2.1. Prepare 4% agar (e.g., 2 g of agar in 50 mL of water) by dissolving agar powder into purified water that has been filtered to remove trace metals and other impurities. Heat the mixture in a microwave until it just begins to boil (approximately 30–60 s). Pour the molten agar into a mold and allow it to cool and solidify at 4 ˚C in a fridge at an approximately 12˚ incline.

NOTE: For a mold, one can use any glass or plastic container set at an angle, with enough molten agar poured in to form a ramp of 4 cm in length and approximately 0.8 cm in height.

3. Stage the slicing area

3.1. Fill a 400 mL beaker containing a slice recovery chamber with approximately 250 mL of ACSF; place it in a water bath warmed to 32 ˚C and begin bubbling with carbogen (95% oxygen gas/5% carbon dioxide gas).

NOTE: Fill the recovery beaker with just enough fluid to place the nylon mesh of the holding chamber at the very surface of the solution, so that slices will be held at the interface of the warm solution mixture and the air (**Figure 1**). Place small pieces of lens paper on the nylon mesh. The slices will lay on top of the lens paper, which can later be used to transfer slices individually to the recording chamber (see step 6.6).

3.2. Place a flask with the sucrose solution into an ice bucket to chill and begin bubbling with carbogen.

NOTE: The recovery beaker and sucrose solution should be warmed and chilled, respectively, with carbogen bubbling for at least 30 mins before the mouse is placed in the isoflurane chamber.

3.3. Pull out the tray of premade, frozen sucrose solution ice cubes from the freezer and place

on the bench to partially thaw.

3.4. Place one half of a clean double-edged razor blade into the microtome and calibrate if necessary. Set aside the other half of the razor blade for use during the dissection.

3.5. Run a carbogen line and temperature probe into the slicing chamber and surround with ice to chill the chamber.

3.6. Prepare a clean bench or table covered with two disposable absorbent pads. Lay out all dissection tools and three pieces of lab tape on the left pad, where the transcardial perfusion will be performed.

NOTE: Dissection tools include small Bonn scissors, dissector scissors, large spatula/spoon, microspatula, scalpel with new blade, spatula, sharp/blunt surgical scissors, large tissue forceps, and small tissue forceps (see **Table of Materials**).

3.7. On the other absorbent pad to the right of the perfusion area, place a circular piece of filter paper into the lid of a 100 mm diameter glass Petri dish. Place the bottom of the Petri dish next to the lid and place a carbogen line into both pieces of the dish.

3.8. Use a razor blade or scalpel to cut a small ramp of agar (approximately 4 cm along the angled surface, 0.8 cm in height, 2 cm in width). Cut off a piece of the ramp from the tall end and reverse it to create a backing block of agar. Use cyanoacrylate adhesive to affix the agar ramp and backing block to the slicing platform and set aside.

NOTE: The backing block of agar helps stabilize the brain during slicing and provides a surface on which to dissect away unnecessary tissues from each slice (**Figure 1**).

4. Transcardial perfusion

4.1. Suspend a 60 mL capacity syringe as a reservoir for sucrose cutting solution approximately 18 inches above the benchtop (e.g., using a three-pronged swivel clamp on a vertical post). Attach tubing to the bottom of the reservoir, run the tubing through a roller clamp, and connect the other end of the tube to a clean 20 G needle.

4.2. Fill the 60 mL reservoir with approximately 30 mL of chilled sucrose solution and direct a carbogen line into the sucrose reservoir to continually bubble the perfusate.

NOTE: Ensure that the sucrose is flowing through the tubing and needle without any trapped bubbles. The flow rate should be just fast enough to have a steady, continual stream of dripping sucrose from the needle.

4.3. Using a blender, crush and blend the frozen sucrose into an icy slurry and use the large spatula/spoon to distribute the slurry.

4.3.1. Place a small amount of sucrose slurry around the edges of the glass Petri dish lid. Add a small amount of sucrose slurry to the Petri dish bottom.

4.3.2. Add roughly 20–30 mL of sucrose slurry to the perfusion fluid reservoir, mixing it well with the 30 mL of chilled liquid sucrose until the reservoir contains a mixture of very cold, predominantly liquid sucrose with some remnant frozen solution.

4.4. Place the mouse into a chamber connected to an isoflurane vaporizer. With oxygen flowing at approximately 2 L/min, turn the dial of the vaporizer to deliver isoflurane at 5% concentration and start a timer.

NOTE: Keep this timer going throughout the course of slicing to gauge how quickly the slices are obtained. The procedure should be done as quickly as possible, such that slicing is complete, and tissue is recovering in the interface chamber within 15–20 min of the animal entering the isoflurane chamber. Watch the mouse to ensure a state of deep anesthesia is achieved. After a minimum of 5 min, the mouse should be deeply anesthetized and unresponsive to toe pinches.

4.5. Immediately before removing the mouse from the isoflurane chamber, fill the Petri dish lid with chilled sucrose solution to a depth of approximately 3–5 mm and fill the Petri dish bottom with chilled sucrose solution to a depth of approximately 1.0 cm.

4.6. Quickly transfer the mouse to the left absorbent pad and use the 3 pieces of tape to secure the forelimbs and tail. Using the large tissue forceps and surgical scissors, tent the skin and make a lengthwise incision from the bottom of the sternum to the top of the chest. Using the forceps pull up on the sternum and use the scissors to cut through the diaphragm.

NOTE: The initial positioning of the mouse and first several incisions to sever the diaphragm should be performed as quickly as possible to ensure the mouse does not regain consciousness.

4.7. Use the scissors to cut through the rib cage on each side, cutting in one large motion towards the point where the forelimb meets the body. With the forceps, swing the front of the rib cage away and up toward the head, and then fully remove it with a horizontal cut using the large scissors. Hold the heart in place using the large forceps and insert the 20 G perfusion needle into the left ventricle. Once the needle is positioned correctly, the left side of the heart should quickly pale as chilled sucrose solution fills the ventricle.

4.8. Use the small dissector scissors to cut into the right atrium and allow the blood to flow out of the circulatory system. If the incisions are performed correctly, there should be minimal damage to the heart, and it should continue pumping throughout the perfusion.

NOTE: Rolled-up pieces of tissue paper can be used to wick away blood and sucrose solution from the heart and maintain visibility of the perfusion needle during the procedure.

4.9. Ensure that the perfusion needle stays in place and does not fall out of the left ventricle. With a proper flow rate and placement, the liver will start to pale to a light tan/beige color with 20–30 s.

4.10. After 30–45 s, use the large scissors to decapitate the mouse. Holding the head in the left hand, push or peel the skin away from the skull. In a well-perfused animal, the skull should be very pale, and the brain should appear a very light pink color (approaching white) through the skull without clearly visible blood vessels.

5. Extract the brain and cut slices

5.1. Using the small Bonn scissors, make two lateral cuts through the skull towards the midline at the front of the skull, near the eyes. Make two additional cuts on either side of the base of the skull.

5.2. Transfer the head to the bottom of the glass Petri dish where it should be mostly immersed in chilled sucrose solution. Use the small Bonn scissors to cut down the midline along the length of the skull, pulling up with the scissors to minimize damage to the underlying brain tissue. Using the small tissue forceps, firmly grasp each side of the skull and swing it up and away from the brain, like opening a book.

5.3. Use the fingers of the left hand to hold the flaps of skull open and insert the microspatula under the brain near the olfactory bulbs. Flip the brain out of the skull into the sucrose and use the microspatula to sever the brain stem. Wash of the brain to remove any residual blood, fur, or other tissues.

5.4. Use the large spatula/spoon to transfer the brain to the lid of the glass Petri dish. With the other half of the double-edged razor blade previously set aside, make two coronal cuts to first remove the cerebellum and then the most anterior portion of the brain, including the olfactory bulbs (**Figure 1**).

5.5. Apply cyanoacrylate adhesive to the agar ramp. Very briefly place the brain onto a piece of dry filter paper using the large tissue forceps and then immediately transfer it to the agar ramp, placing the ventral surface of the brain onto the adhesive.

NOTE: For slices of the intermediate hippocampus, the brain should be oriented with the anterior facing up the slope of the ramp, and the posterior closer to the blade. For slices of the ventral hippocampus, orient the brain with the anterior end pointed down the slope of the ramp, with the posterior end at the top of the ramp, further away from the blade. In either case, the brain should be positioned at the top of the ramp, such that the coronally-cut surface of the brain contacts the agar backing block (**Figure 1**).

5.6. Place the slicing platform with the agar and brain into the slicing chamber of the microtome and completely immerse with chilled sucrose solution. Use the large spatula/spoon

to transfer some sucrose slurry to the chamber, stirring to melt any frozen sucrose and rapidly bring down the temperature of the mixture to ~1–2 ˚C.

NOTE: Watch the temperature gauge throughout the slicing. If the sucrose solution warms above 3 ˚C, add more slurry and mix to bring the temperature back down.

5.7. Cut slices at a thickness of 450 µm with the microtome speed set to 0.07 mm/s. As each slice is freed, use the small tissue forceps and a sharp scalpel to first separate the two hemispheres, and then to cut away tissue until the slice consists primarily of the hippocampus and parahippocampal regions (**Figure 1**).

5.8. Use a plastic transfer pipette to transfer the slices individually to the interface recovery chamber, ensuring that they are positioned at the interface of the ACSF and the air with only a thin meniscus of ACSF covering the slices. Tightly close the lid of the chamber and allow slices to recover at 32 ˚C for 30 min.

5.9. After the initial recovery at 32 ˚C, bring the interface recovery chamber out of the water bath and place on a stirrer set to a slow speed such that the magnetic stir bar promotes circulation of ACSF within the chamber. Allow it to gradually cool to room temperature as the slices recover for an additional 90 min. Ensure that the recovery chamber is continually bubbled with carbogen and do not allow large bubbles to become trapped underneath the slices.

6. Perform local field potential (LFP) recordings of spontaneous activity

6.1. Prepare for LFP recordings by turning on all necessary equipment, including the computer running the acquisition software, the monitor connected to the computer, the stimulators, the micromanipulator, the temperature controller, the microscope light source, the microscope-attached camera, the microelectrode amplifier, the digitizer, and the peristaltic pump. If using a central vacuum system, open the wall valve to prepare the vacuum line that will remove ACSF from the recording chamber.

6.2. Fill the heated reservoir with ACSF, then place one end of the tubing into the 400 mL beaker containing the bubbling ACSF. Turn on the peristaltic pump to direct ACSF from the 400 mL beaker to the heated reservoir, and from the reservoir onward to the recording chamber. Tap or pinch the tubing to release any trapped bubbles.

6.3. Adjust the peristaltic pump to ensure that the ACSF flow rate through the recording chamber is fast (~ 8–10 mL/min). Use a temperature probe to ensure that the ACSF is 32 ˚C in the center of the recording chamber.

NOTE: If ACSF is delivered to the recording chamber using a peristaltic pump, a high flow rate can result in a significant fluctuation in flow rate. Consistent flow rates can be achieved using a simple pulsation dampener consisting of a series of empty syringes integrated into the tubing **(Figure 2)**.

6.4. Prepare stimulation and recording pipettes from borosilicate capillaries using a heated filament puller. The puller protocol should be configured to yield pipettes with a resistance of 2–3 MOhm for stimulation or local field potential recording electrodes.

6.5. Fill stimulation pipettes with 1 M NaCl and LFP pipettes with ACSF.

6.6. Briefly clamp the tubing and turn off the pump to pause flow of ACSF. Transfer a slice to the recording chamber using fine forceps to grasp a corner of the lens paper the slice is resting on-the slice will stick to the lens paper. Place the lens paper and slice into the recording chamber with the slice facing down, then "peel" away the lens paper leaving the slice submerged in the recording chamber. Secure the slice using a harp.

NOTE: Harps can be purchased pre-made or made in-lab using a U-shaped piece of stainless steel or platinum and fine nylon filament.

6.7. Place a NaCl-filled stimulation pipette in the manual micromanipulator and slowly advance the tip of the pipette into the surface of the slice (e.g., in the SR layer) at an angle of approximately 30–45˚. Once the tip of the pipette enters the tissue, advance the pipette forward slowly and refrain from large movements in the lateral or vertical direction that could unnecessarily damage axons within the slice. Place the tip of the pipette at least 50–100 μm deep into the slice to avoid cells near the surface that were damaged during slicing.

6.8. Place an ACSF-filled LFP pipette into the pipette holder attached to the motorized micromanipulator. Apply a very light positive pressure using either mouth pressure or a 1 mL syringe connected via a stopcock valve and a short length of tubing to the pipette holder.

NOTE: Position LFP pipettes within the slice in order to record the signals of interest: in the case of sharp wave ripples, one LFP pipette should be placed in the *stratum pyramidale* (SP) and a second pipette in the *stratum radiatum* (SR). This configuration allows for simultaneous recordings of the negative sharp-wave in the SR and the high-frequency ripple oscillation in the SP (**Figure 3**).

6.9. Using the micromanipulator slowly advance the tip of the LFP pipette into the region of interest (e.g., the CA1 pyramidal cell layer) at an angle of approximately 30–45˚. Place the tip of the pipette at least 50–100 μm deep into the slice to avoid cells near the surface that were damaged during slicing. While advancing the LFP pipette, continually deliver a small voltage test pulse using the acquisition software and watch for a sudden increase in electrode resistance. This may indicate that the pipette has been clogged or pressed up against a cell.

6.10. Once the LFP pipette is positioned in the region of interest, carefully release the positive pressure by opening the valve on the tubing attached to the pipette holder.

6.11. To record the LFP in a second location simultaneously, repeat steps 6.8–6.10 with a second pipette and micromanipulator.

6.12. Use the microelectrode amplifier in the current clamp configuration to record spontaneous activity in the local field potential after using the bridge balance function in the acquisition software to correct for series resistance of the pipette. Spontaneous SWRs will appear as positive deflections in the extracellular potential of the SP layer (**Figure 3**).

6.13. In order to record evoked field potentials, use the stimulators connected to the digitizer to deliver a short (200 us) square voltage pulse through the NaCl-filled stimulation pipette. Adjust the stimulation voltage dial to evoke a range of responses amplitudes.

REPRESENTATIVE RESULTS:

Presented here are representative recordings from HEC slices prepared as described in this protocol. Following recovery in an interface holding chamber (**Figure 1C**), slices are transferred individually to a submerged recording chamber (**Figure 2B**). The recording chamber is supplied with carbogen-saturated ACSF using a peristaltic pump (**Figure 2A**). The pump first draws ACSF from a holding beaker into a heated reservoir. Carbogen lines are placed into both the holding beaker and the heated reservoir to provide continuous oxygenation of the media. A pulsation dampener, consisting of a series of air-filled syringes, is positioned in between the peristaltic pump and the recording chamber to minimize the fluctuations in flow rate produced by rapid peristalsis. The air pocket in each syringe absorbs the changes in pressure caused by each cycle of the pump, so that the recording chamber receives a smooth and consistent flow of ACSF[52,53]. An inline heater positioned after the pulsation dampener ensures that the temperature of the ACSF is held at 32 °C as it enters the recording chamber.

In this example, the dual-surface superfusion recording chamber consists of three 3D-printed layers (**Figure 2B**). The bottom layer has a rectangular cutout to fit a coverslip, secured with vacuum grease. The middle layer contains the bottom half of an elongated oval chamber, with two horizontal supports. Nylon filament is strung across these supports (roughly every 0.5 mm) and secured with cyanoacrylate adhesive. The slice will rest on top of this strung filament. The top layer contains the upper half of the oval chamber along with small wells into which the silver chloride ground pellets can be placed. The elongated oval shape of the chamber is designed to promote fast laminar flow of ACSF.

Figure 3 presents representative recordings from HEC slices prepared according to this protocol. To initially assess slice health, field postsynaptic potentials (fPSPs) are evoked in the *stratum radiatum* (SR) using a pipette filled with 1 M of NaCl. In healthy slices, electrical stimulation should produce a fPSP with a small presynaptic fiber volley and a large postsynaptic potential with a rapid initial descent (**Figure 3B, upper left**). In healthy slices, spontaneous sharp-wave ripples (SWRs) are visible as positive deflections in the LFP in the *stratum pyramidale* (**Figure 3B, lower left**). In suboptimal slices evoked fPSPs show a large fiber volley and a relatively small postsynaptic potential, and such slices do not show spontaneous SWRs (**Figure 3B, right**). SWRs in vitro show characteristics consistent with published descriptions: a positive field potential in the SP layer with an overlaid high frequency oscillation, paired with a negative field potential in the SR layer (**Figure 3C**). A single SWR recorded in CA2 is indicated with an asterisk (**Figure 3C,**

right). SWRs in HEC slices originate within CA2/CA3 recurrent circuits and propagate to CA1. A single SWR observed in the CA2 and CA1 SP layer is indicated with an asterisk (**Figure 3D, right**). In this representative example, the CA2 SWR (green) leads that in CA1 (blue) by several milliseconds, as shown in the overlay of the SWR envelope (filtered at 2–30 Hz) recorded in each region.

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FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of horizontal angled hippocampal-entorhinal cortex (HEC) slices. (**A**)(**i**) After extracting the brain, perform two coronal cuts with a razor blade to remove the posterior and anterior portions of the brain. (**ii**) The agar ramp is formed of two angled portions glued to the microtome slicing platform. To prepare slices of the intermediate hippocampus, place the brain block onto the agar ramp with the anterior surface facing up the slope and making contact with the tall backing portion of the ramp. To prepare slices of more ventral hippocampus, place the brain block onto the agar ramp with the anterior surface facing down the slope, so that the posterior cut surface makes contact with the tall backing portion of the ramp. (**iii**) As each slice is freed, perform several more cuts with the scalpel to separate the hemispheres and remove unnecessary tissue. (**B**) Representative image of the resulting slice with cell nuclei labeled by DAPI. (**C**) In an interface recovery, chamber slices are placed on pieces of lens paper on top of a stainless steel or nylon mesh, level with the surface of the ACSF. A ceramic bubbler conveys carbogen into the chamber and a magnetic stir bar continually mixes the fluid in the chamber. A thin film of ACSF covers the top surface of the slice, enhancing diffusion of oxygen from the humid carbogen-rich air of the chamber.

Figure 2: Dual surface superfusion recording chamber with pulsation dampener in the ACSF delivery tubing. (**A**) Diagram of the superfusion system. ACSF is warmed to 32 ˚C, constantly bubbled with carbogen gas, and delivered at approximately 8–10 mL/min using a peristaltic pump with a pulsation dampener consisting of a series of air-filled syringes. (**B**) The recording chamber consists of three 3D-printed layers, the middle of which is strung with nylon filament. The slice rests upon this strung filament and ACSF flows above and below the tissue.

Figure 3: Representative recordings of spontaneous sharp-wave ripples from HEC slices. (**A**) A simplified diagram of the HEC slice showing the positions of the recording and stimulation electrodes. (**B**) Representative recordings of LFP activity from both an active, healthy slice and a suboptimal slice. The healthy slice (left, in green) shows large evoked field responses and

spontaneous sharp-wave ripples (SWRs), visible as irregularly occurring positive deflections in the local field potential of the SP layer. In contrast, an unhealthy slice shows small evoked field responses and no spontaneous activity (right, in gray). (**C**) Representative recordings of SWRs in the CA2 region, consisting of a negative deflection in the LFP in the SR layer and a high frequency oscillation with an underlying positive deflection in the LFP in the SP layer. Peaks in each channel greater than three standard deviations of the signal amplitude are highlighted in red. A bandpass filter of 2–30 Hz isolates the underlying positive and negative envelope of the sharp wave in the SP and SR layer, respectively, while a bandpass filter of 80–250 Hz is used to isolate the high-frequency oscillation of the ripple in the SP layer. (D) SWRs in vitro propagate from CA2/CA3 to CA1. In these representative recordings, SWRs in CA2 (green, bottom) precede that in CA1 (blue, top) by several milliseconds. Peaks in each channel greater than three standard deviations of the signal amplitude are highlighted in red.

Table 1: Composition of sucrose cutting solution. Begin with approximately 0.75 L of purified water that has been filtered to remove trace metals and other impurities. Dissolve each solid while mixing the solution with a magnetic stir bar. Once all solids are dissolved, bubble carbogen gas through the solution for 10 min. Add the $MgCl_2$ and $CaCl_2$ solutions and add water to bring the total volume to 1 L. Mix with a magnetic stir bar for 10 min to ensure the solution is uniformly mixed. The osmolarity should be between 315 and 325 mOsm, and the pH should be approximately 7.4.

Table 2: Composition of artificial cerebrospinal fluid. Begin with approximately 1.5 L of purified water that has been filtered to remove trace metals and other impurities. Dissolve each solid while mixing the solution with a magnetic stir bar. Once all solids are dissolved, bubble carbogen gas through the solution for 10 min. Add the $MgCl_2$ and $CaCl_2$ solutions and add water to bring the total volume to 2 L. Mix with a magnetic stir bar for 10 min to ensure the solution is uniformly mixed. The osmolarity should be between 315 and 325 mOsm, and the pH should be approximately 7.4.

DISCUSSION:

There are several steps in this slicing protocol designed to promote tissue health and favor the emergence of spontaneous naturalistic network activity: the mouse is transcardially perfused with chilled sucrose cutting solution; horizontal-entorhinal cortex (HEC) slices are cut at a thickness of 450 μm from the intermediate or ventral hippocampus; slices recover at the interface of warmed ACSF and humidified, carbogen-rich air; during recordings slices are superfused with ACSF warmed to 32 °C and delivered at a fast flow rate with dual-surface superfusion in a submerged recording chamber.

Slice health is of paramount importance for the generation of network oscillations in vitro. Young animals will yield more healthy slices, and generally with juvenile or adolescent mice the transcardial perfusion step can be skipped. As animals age, it becomes increasingly difficult to make healthy slices, yet some investigations (such as disease models or longitudinal studies) necessitate the use of adult or aging animals. Dengler et al., for example, used transcardial perfusions in their preparation of HEC slices from pilocarpine-treated chronically epileptic adult

mice[50]. With adult mice, it is beneficial to perform a transcardial perfusion with chilled sucrose cutting solution to cool the tissue, clear blood from the brain, and reduce metabolic activity before removing the brain from the skull[51]. It is important to note, however, that transcardial perfusions require training to be performed correctly and care must be taken to ensure that the procedure is carried out quickly and in a way that does not pose a risk to animal welfare. When possible, experiments should be designed to use young animals so as to preclude the use of transcardial perfusions. In a suboptimal slice preparation, there will not be enough healthy cells, particularly interneurons, to support ongoing network oscillations. To assess slice health during the experiment, it is useful to first record evoked field potentials (for example, with a stimulation pipette and a recording pipette in the *stratum radiatum*, SR). In a healthy slice, stimulation in the SR layer will evoke a large field postsynaptic potential (fPSP) with a relatively small presynaptic fiber volley (**Figure 3**).

The slices produced by this protocol are angled horizontal hippocampal-entorhinal cortex (HEC) slices. Importantly, neither the inclusion of parahippocampal tissue nor the angled cut are necessary for hippocampal slices to spontaneously generate network activities such as SWRs. Indeed, many studies have utilized horizontal or transverse hippocampal slices to interrogate aspects of physiological[25,40,41,44] or pathological network oscillations[33,38]. In this protocol the placement of the brain onto an agar ramp allows the experimenter to selectively produce more slices from either the ventral or intermediate hippocampus (**Figure 1**), which may be beneficial for experimental objectives that take into account the functional heterogeneity that exists along the longitudinal axis of the hippocampus[26,31]. If the anatomical origin of the slices is not a factor, then the agar ramp can be excluded and a true horizontal cutting plane will yield slices of the intermediate-to-ventral hippocampus. As each horizontal tissue slice is freed, most extraneous parts of the slice can be removed with three simple cuts, leaving a roughly rectangular slice that contains the hippocampus and some surrounding tissue, including the parahippocampal region (**Figure 1**). Further dissection can be performed to remove all extrahippocampal tissues, but the inclusion of surrounding tissue is beneficial, in that, the slice harp can be easily placed such that the nylon filaments do not rest across the hippocampus proper. As discussed above, the combined HEC slice is also a useful preparation with which to investigate a larger corticohippocampal network in the context of physiological[37] or pathological[16,35,36,38] network oscillations.

The key factor in this protocol is to optimize oxygen supply to the tissue, both during the recovery phase and during the recordings. Many studies of network oscillations are performed in slices that are transferred directly from the microtome to an interface recording chamber and allowed to recover with continual perfusion of fresh ACSF. After several hours of recovery, recordings can then be performed in the same interface chamber. Thus, slices are held at the interface of ACSF and humid carbogen-rich air for the full duration of the experiment. In the alternative methodology presented in this protocol, slices recover in an interface-style holding chamber for at least two hours before individual slices are transferred to a submerged-style recording chamber with sufficiently fast ACSF flow rates. Slices prepared under these conditions can exhibit stable gamma oscillations[12] or spontaneous SWR activity[43]. Slice recovery in an interface-style holding chamber is a critical step: Maier et al. demonstrated that slices which recover in a beaker

completely submerged in ACSF exhibit smaller evoked field potentials, less frequent spontaneous postsynaptic currents, and only rarely produce spontaneous network activity[43]. Similarly, Hájos et al. demonstrated that fast ACSF flow rates result in a higher frequency of spontaneous inhibitory postsynaptic currents, suggesting improved interneuronal activity[49]. During the recording period, dual-surface superfusion is not strictly necessary, provided the recording chamber holds a relatively small volume of ACSF delivered at a fast flow rate (at least 6 mL/min)[43]. The 3D printed recording chamber presented in this protocol (**Figure 2B**) is a relatively cost effective and a simple option, but there are also commercially available submerged recording chambers designed to hold smaller volumes, promote laminar flow of the media, or provide dual-surface superfusion (in contrast to circular recording chambers, for example, which allow excess dead volume of ACSF).

While this protocol allows one to record network oscillations without the requirement of a traditional interface recording chamber, there are several limitations. Although slices are held in the ACSF-air interface during the recovery period, they do not receive continual perfusion of fresh media as occurs in traditional Haas-style interface recording chambers. Slices must be transferred individually (using a plastic transfer pipette) from the recovery chamber to the submerged recording chamber. Furthermore, fast flow rates can cause instability and motion artifacts problematic for some recordings, particularly if ACSF is delivered with a peristaltic pump. In order to maintain fast flow rates and minimize mechanical disturbances, a simple pulsation dampener can be integrated into the perfusion system (**Figure 2**). This pulsation dampener operates using the Windkessel effect[52], in which empty syringes contain air pockets that act as elastic reservoirs, absorbing the fluctuating pressure generated by the rollers of the peristaltic pump[53]. However, incorporation of a pulse dampener can add length to the tubing that delivers ACSF to the recording chamber and impact oxygen supply to the slice. Flow rates should only be as fast as is necessary to yield stable network oscillations, and if a peristaltic pump is used it should ideally be a pump that uses a large number of rollers (> 12) to minimize the pulsation caused by peristalsis, preclude the use of a pulsation dampener, and ensure that the tubing that delivers ACSF to the recording chamber is as short as possible. Recordings performed with fast flow rates also necessitate large volumes of ACSF, which may be problematic if experiments require the addition of valuable or expensive drugs or compounds to the media. Although a dual-surface superfusion chamber requires a specially constructed recording chamber with two fluid inlets to deliver oxygenated media to both sides of the slice, spontaneous network oscillations can be observed with moderate ACSF flow rates[48]. This protocol utilizes both a fast flow rate (stabilized with a pulsation dampener) and a dual-surface superfusion chamber to improve the likelihood of observing spontaneous activity.

Finally, this protocol has a low yield with regards to the number of slices produced per animal. Horizontal slicing with a thickness of 450 μm yields a small number of HEC slices at the preferred orientation (in which the slice is effectively parallel to the transverse axis of the hippocampus). Of these slices, typically, only one or two per hippocampi exhibits spontaneous SWR activity, fewer than has been reported elsewhere[43]. Though thicker slices presumably contain a greater degree of recurrent connectivity, cutting slightly thinner slices (400 μm) may yield a greater number per mouse of transversely-oriented slices with SWR activity. In addition, the likelihood

that multiple slices reliably exhibit spontaneous SWR activity may be higher in experiments that utilize true horizontal or downward angled slices of the ventral hippocampus. The current protocol incorporates the use of upward angled horizontal slices of the intermediate hippocampus, which may be less likely to exhibit spontaneous network activity in comparison to slices from the ventral hippocampus[25,26,31]. Finally, this protocol used slices from adolescent and adult mice. While transcardial perfusions can improve the quality of slices from older animals, the likelihood of observing spontaneous network activities may be improved by the use of slices from younger animals[12,41,44].

In summary, this protocol presents a mouse brain slicing approach that yields angled horizontal hippocampal-entorhinal cortex slices from the intermediate or ventral hippocampal formation that can exhibit complex spontaneous network activity in the form of sharp wave-ripple complexes.

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DISCLOSURES:

The author has nothing to disclose.

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Figure 1

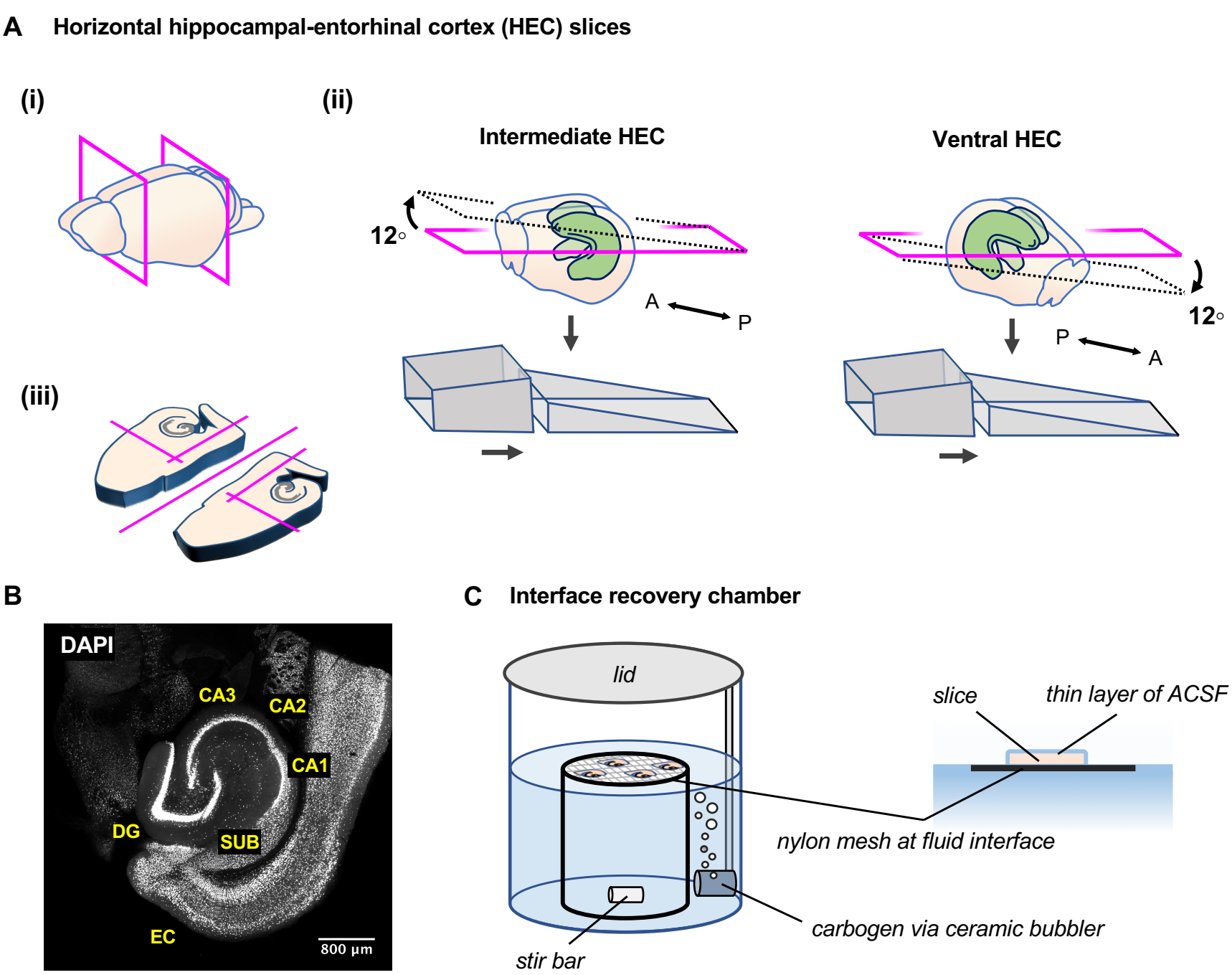

Figure 2

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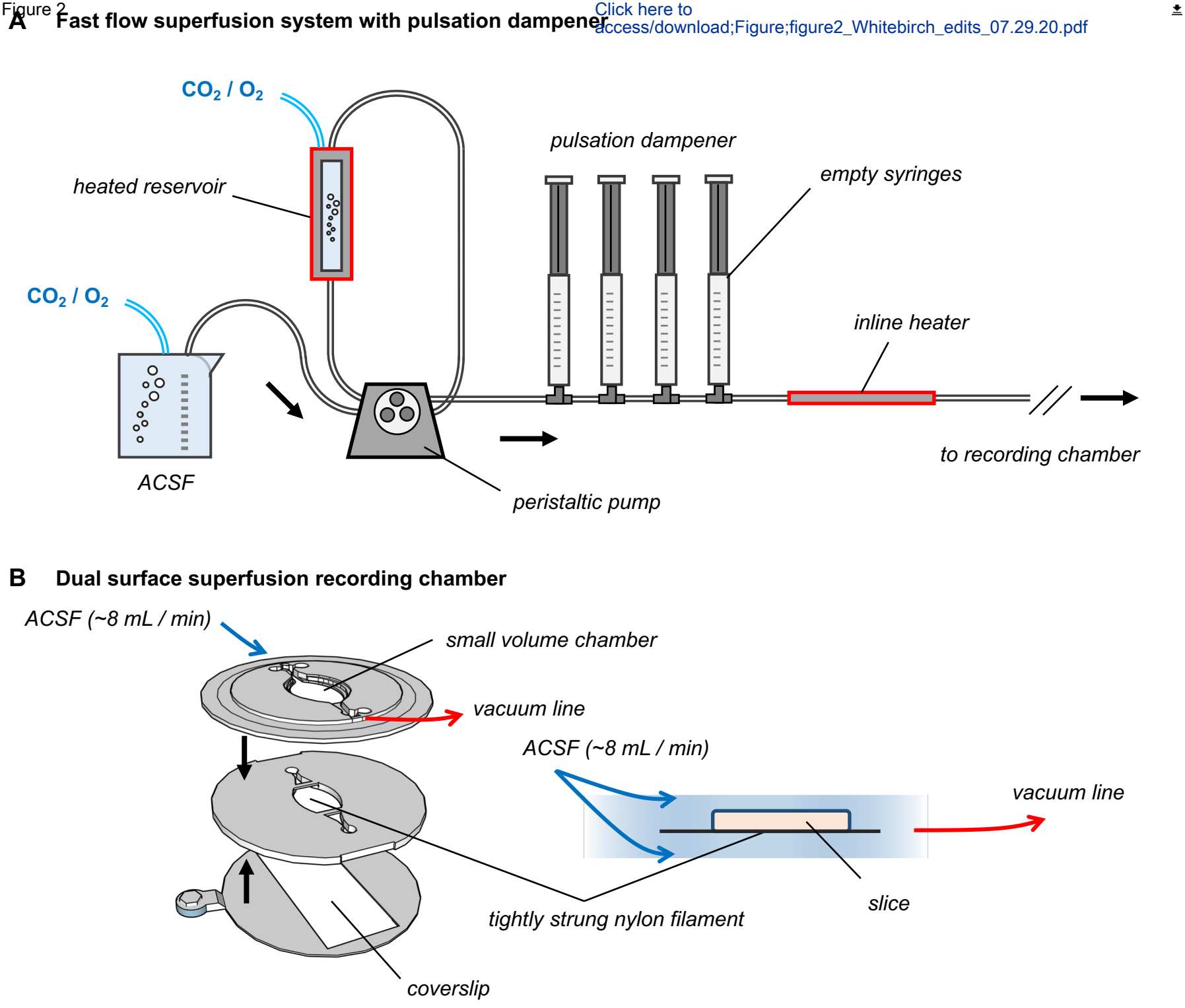

Figure 3

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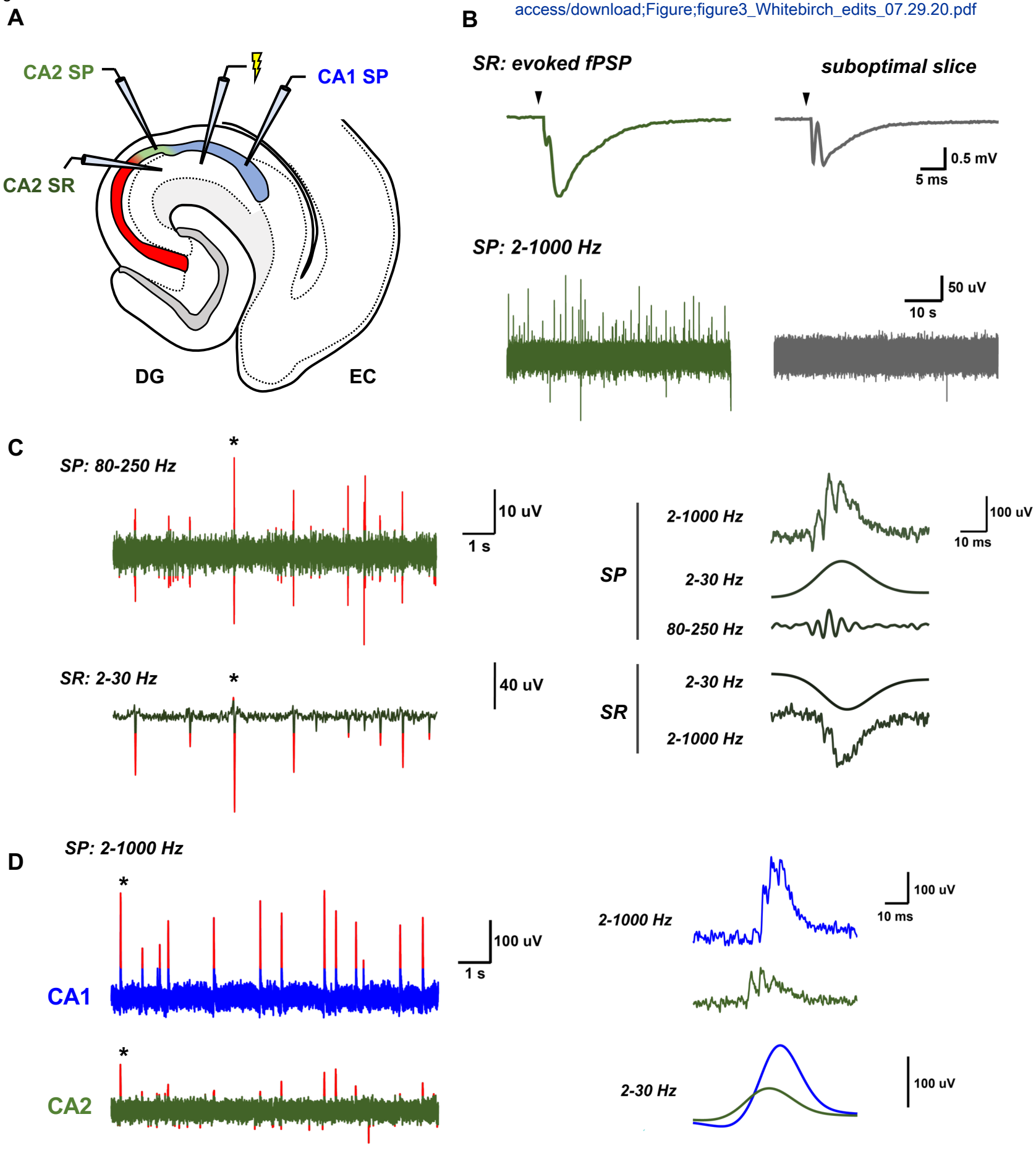

Table

		molecular weight (grams / mol)	final concentration (mM)	grams / 1 L sucrose cutting solution
sucrose	$C_{12}H_{22}O_{11}$	342.3	195	66.749
sodium chloride	NaCl	58.44	10	0.584
glucose	$C_6H_{12}O_6$	180.08	10	1.801
sodium bicarbonate	$NaHCO_3$	84.01	25	2.1
potassium chloride	KCl	74.55	2.5	0.186
sodium phosphate monobasic anhydrous	NaH_2PO_4	137.99	1.25	0.173
sodium pyruvate	$C_3H_3NaO_3$	110.04	2	0.22

		stock concentration (M)	final concentration (mM)	milliliters / 1L sucrose cutting solution
calcium chloride	$CaCl_2$	1	0.5	0.5
magnesium chloride	$MgCl_2$	1	7	7

Table

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		molecular weight (grams / mol)	final concentration	grams / 2L ACSF
sodium chloride	NaCl	58.44	125	14.61
glucose	$C_6H_{12}O_6$	180.08	12.5	4.502
sodium bicarbonate	$NaHCO_3$	84.01	25	4.201
potassium	KCl	74.55	3.5	0.522
sodium phosphate monobasic anhydrous	NaH_2PO_4	137.99	1.25	0.345
ascorbic acid	$C_6H_8O_6$	176.12	1	0.352
sodium pyruvate	$C_3H_3NaO_3$	110.04	3	0.66
		stock concentration (M)	final concentration (mM)	milliliters / 2L ACSF
calcium chloride	$CaCl_2$	1	1.6	3.2
magnesium chloride	$MgCl_2$	1	1.2	2.4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3D printer	Lulzbot	LulzBot TAZ 6	
Acute brain slice incubation holder	NIH 3D Print Exchange	3DPX-001623	Designed by ChiaMing Lee, available at https://3dprint.nih.gov/discover/3dpx-001623
Adenosine 5'-triphosphate magnesium salt	Sigma Aldrich	A9187-500MG	
Ag-Cl ground pellets	Warner	64-1309, (E205)	
agar	Becton, Dickinson	214530-500g	
ascorbic acid	Alfa Aesar	36237	
beaker (250 mL)	Kimax	14000-250	
beaker (400 mL)	Kimax	14000-400	
biocytin	Sigma Aldrich	B4261	
blender	Oster	BRLY07-B00-NP0	
Bonn scissors, small	becton, Dickinson	14184-09	
borosilicate glass capillaries with filament (O.D. 1.5 mm, I.D. 0.86 mm, length 10 cm)	Sutter Instruments	BF150-86-10HP	Fire polished capillaries are preferable.
calcium chloride solution (1 M)	G-Biosciences	R040	
camera	Olympus	OLY-150	
compressed carbogen gas (95% oxygen / 5% carbon	Airgas	X02OX95C200310	
compressed oxygen	Airgas	OX 200	
constant voltage isolated stimulator	Digitimer Ltd.	DS2A-Mk.II	
coverslips (22x50 mm)	VWR	16004-314	
cyanoacrylate adhesive	Krazy Glue	KG925	Ideally use the brush-on form for precision
data acquisition software	Axograph	N/A	Any equivalent software (e.g. pClamp) would work.

Dell Precision T1500 Tower Workstation Desktop	Dell	N/A	Catalog number will depend on specific computer - any computer will work as long as it can run electrophysiology acquisition software.
Digidata 1440A	Molecular Devices	1-2950-0367	
digital timer	VWR	62344-641	4-channel Traceable timer
disposable absorbant pads	VWR	56616-018	
dissector scissors	Fine Science Tools	14082-09	
double-edge razor blades	Personna	BP9020	
dual automatic temperature controller	Warner Instrument	TC-344B	
dual-surface or laminar-flow optimized recording chamber	N/A	N/A	The chamber presented in this protocol is custom made. A commercial equivalent would be the RC-27L from Warner Instruments.
equipment rack	Automate Scientific	FR-EQ70"	A rack is not strictly necessary but useful for organizing electrophysiology
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	Sigma Aldrich	324626-25GM	
filter paper	Whatman	1004 070	
fine scale	Mettler Toledo	XS204DR	
Flaming/Brown micropipette puller	Sutter Instruments	P-97	
glass petri dish (100 x 15 mm)	Corning	3160-101	
glucose	Fisher Scientific	D16-1	
Guanosine 5′-triphosphate sodium salt hydrate	Sigma Aldrich	G8877-250MG	
ice buckets	Sigma Aldrich	BAM168072002-	
isoflurane vaporizer	General Anesthetic	Tec 3	

lab tape	Fisher Scientific	15-901-10R	
lens paper	Fisher Scientific	11-996	
light source	Olympus	TH4-100	
magnesium chloride solution (1 M)	Quality Biological	351-033-721EA	
magnetic stir bars	Fisher Scientific	14-513-56	Catalog number will be dependent on the size of the stir bar.
micromanipulator	Luigs & Neumann	SM-5	
micromanipulator (manual)	Scientifica	LBM-2000-00	
microscope	Olympus	BX51WI	
microspatula	Fine Science Tools	10089-11	
monitor	Dell	2007FPb	
MultiClamp 700B Microelectrode Amplifier	Molecular Devices	MULTICLAMP 700B	The MultiClamp 700B should include headstages, pipette holders, and a model cell.
N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic	Sigma Aldrich	H3375-25G	
needle (20 gauge, 1.5 in length)	Becton, Dickinson	305176	
nylon filament	YLI Wonder Invisible Thread	212-15-004	size 0.004. This cat. # is from Amazon.com
nylon mesh	Warner Instruments	64-0198	
perstaltic pump	Harvard Apparatus	70-2027	
Phosphocreatine di(tris) salt	Sigma Aldrich	P1937-1G	
pipette holders	Molecular Devices	1-HL-U	
platinum wire	World Precision	PT0203	
polylactic acid (PLA) filament	Ultimaker	RAL 9010	
potassium chloride	Sigma Aldrich	P3911-500G	
potassium gluconate	Sigma Aldrich	1550001-200MG	
potassium hydroxide	Sigma Aldrich	60377-1KG	
razor blades	VWR	55411-050	

roller clamp	World Precision	14041	
scale	Mettler Toledo	PM2000	
scalpel handle	Fine Science Tools	10004-13	
slice harp	Warner	SHD-26GH/2	
sodium bicarbonate	Fisher Chemical	S233-500	
sodium chloride	Sigma Aldrich	S9888-1KG	
sodium phosphate monobasic anhydrous	Fisher Chemical	S369-500	
sodium pyruvate	Fisher Chemical	BP356-100	
spatula	VWR	82027-520	
spatula/spoon, large	VWR	470149-442	
sterile scalpel blades	Feather	72044-10	
stirrer / hot plate	Corning	6795-220	
stopcock valves, 1-way	World Precision	14054	
stopcock valves, 3-way	World Precision	14036	
sucrose	Acros Organics	AC177142500	
support for swivel clamps	Fisher Scientific	14-679Q	
surgical scissors, sharp/blunt	Fine Science Tools	14001-12	
syringe (1 mL)	Becton, Dickinson	309659	
syringe (60 mL with Luer-Lok tip)	Becton, Dickinson	309653	
three-pronged clamp	Fisher Scientific	05-769-8Q	
tissue forceps, large	Fine Science Tools	11021-15	
tissue forceps, small	Fine Science Tools	11023-10	
transfer pipettes	Fisher Scientific	13-711-7M	
tubing	Tygon	E-3603	ID 1/16 inch, OD 3/16 inch
tubing	Tygon	R-3603	ID 1/8 inch, OD 1/4 inch
vacuum grease	Dow Corning	14-635-5D	
vibrating blade microtome	Leica	VT 1200S	
vibration-dampening table with faraday cage	Micro-G / TMC-ametek	2536-516-4-30PE	
volumetric flask (1 L)	Kimax	KIM-28014-1000	
volumetric flask (2 L)	PYREX	65640-2000	
warm water bath	VWR	1209	

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July 30th, 2020

In this resubmission of my protocol manuscript, re-titled “An acute mouse brain slicing method for investigation of spontaneous hippocampal network activity”, I have made substantial revisions to incorporate the changes suggested in both the editorial and reviewer comments. I would first like to thank all of the reviewers for their detailed and considerate feedback, and I feel that the manuscript has been greatly improved with these revisions. I have included below specific responses (in blue) to each editorial and reviewer comment.

Sincerely,

Alex C. Whitebirch

Editorial comments:

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

• ***Protocol Language:*** *Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Examples NOT in the imperative: 4.1, 6.1, 6.3, etc.*

I corrected the protocol text to include as much imperative tense as possible (lines 176 - 482).

• ***Protocol Detail:*** *Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video.* ***Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.*** *There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*
1) section 6: please add a few steps to describe stimulation and recording briefly. The protocol seems incomplete as is.

I added several steps to section 6 to include more details for the stimulation and recording

steps. This section is still relatively brief, as I wrote the protocol to emphasize the slicing procedure itself, and the precise nature of the stimulation and recording will vary depending on the user's specific experimental design and objectives (lines 402 - 482).

• ***Protocol Highlight:*** *After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

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

After revision, the total length of the highlighted text is approximately 2.5 pages. Highlighting includes complete sentences and covers the critical steps that should be included in the video (lines 215 - 462).

• ***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 (3-6 paragraphs): 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.*

I heavily revised the manuscript to include a more comprehensive discussion (lines 593 - 705).

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

I updated the list of reference to have unabbreviated journal names (lines 716 - 855).

• *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 figures and tables in this protocol are original and have not been published anywhere.

Reviewers' comments:

Reviewer #1:

This MS, based on its title, proposes a method that may optimize brain slicing for investigation of hippocampal SWRs. The author states in the abstract that hippocampal slices do not typically exhibit SWRs, or other forms of spontaneous network activity. In addition, the author suggests that HEC slices cut with an angle at 450 um thickness preserve more connectivity, so this may be a better approach to study SWRs. First, there is no evidence presented in the MS that this suggestion is valid, i.e. hippocampal slices are less ready to generate spontaneous rhythms. If this is one of the main statements of the MS, then a solid comparison should be done, not only present an example recording without any comparison. Second, this reviewer does not think that hippocampal slices do not typically generates SWRs. Two-three horizontal hippocampal slices per hemisphere typically show SWRs at a thickness of 350-400 um. This is the case even if there is no entorhinal cortex is attached to the hippocampal slices, i.e. the parahippocampal region is not pivotal for SWR generation in vitro. This is in agreement with in vivo data. Therefore, it is hard to see why HEC slices are better to study SWRs than hippocampal slices without the EC, unless this is shown by a systematic comparison. The author may consider the fact that in a 450 um thick slices the connectivity is better preserved than in 400 um thick ones that are usually used in SWR studies, thus the thickness of the slices may be the key factor here and not the presence of EC itself.

My original text was not clearly written in this regard, particularly the statements such as lines 33 & 34 in the original document. The missing context was that this statement was in reference to transverse hippocampal slices of the dorsal hippocampus, maintained under submerged conditions. The abstract and introduction have been heavily revised to clarify this point (lines 26 - 174). Similarly, this manuscript was not intended to make the argument that HEC slices are superior to isolated hippocampal slices in terms of their ability to generate spontaneous SWRs. I revised the text to clarify this issue, and explain that the inclusion of parahippocampal regions is optional and dependent on one's specific experimental objectives (lines 107 – 135; lines 620 - 638). In the original manuscript I was not clear in my rationale for including the angled slice approach. I didn't mean to claim that it is a superior or necessary modification to a "standard" horizontal slice, and you are certainly correct in that this angle is not necessary for the primary objective of producing slices exhibiting spontaneous SWR activity. Indeed many studies I've referenced in this protocol produce spontaneously active slices with no angle. I added a paragraph to clarify the rationale behind the angled horizontal slice, with specific references both to studies that use this slicing angle as well as studies that use a true "flat" horizontal slice (lines 107 - 135; lines 620 - 638). My rationale in including this aspect to the protocol is that the angle can be implemented to preferentially create horizontal slices that parallel the transverse plane of the ventral or intermediate hippocampus. In the original version of figure 1 the brain was depicted with the ventral surface glued to the ramp and the rostral end of the brain

pointed up the slope of the ramp – this “upward” angle, used by Stoop et al., 2000 and Mizunuma et al., 2014, yields more slices from the intermediate hippocampus. I added to the figure a depiction of the alternate angle, where the rostral end of the brain is pointed down the slope (figure 1). With this “downward” angle, the horizontal cut will produce more slices of the ventral hippocampus. The downward angled horizontal slice is used by Jones et al., 1988, Rafiq et al., 1993, Carter et al., 2011, and ul Haq et al., 2016. As with the inclusion of parahippocampal regions in the slice, I added text to clarify that the use of an angled slice is optional and dependent on one’s experimental objectives (lines 123 – 135; lines 620 - 638).

Additional comments:
1)Sections usually refers to thin tissue samples, for instance used for immunostaining. Electrophysiological recordings are done in slices and not in sections. Please revised accordingly.

Good point - all instances of “section” have been replaced with “slice” (lines 29,38,46,137,390,688).

2) In line 90, the given reference should be changed to Hajos et al., 2009.

The reference has been fixed (line 145).

3) Reference Hajos et al., 2004 should be added in line 98, as this is the first demonstration that oscillations can be maintained in submerged slices.

This reference has been added (lines 157,161).

4) In line 101: 'spontaneously active hippocampal tissue'. Tissue is not active, but the hippocampal neurons within the slice are active.

This line has been rewritten (lines 171,172).

5) To maintain SWRs in slices, the author used a very high flow rate in a submerged chamber with dual surface superfusion. This is likely due to the fact that he introduced a series of syringes to dampen pulsation caused by a peristaltic pump. This reviewer assumes that the introduction of this dampener system may lead to a reduction in the oxygen levels, therefore the author needs to apply so fast superfusion rates to maintain SWRs even in dual surface superfusion chamber. If a peristaltic pump with 12 (or more) roller rotors is used, no pulsation disturbs the recordings in the chambers, i.e. no pulsation dampener should be added, thus a shorter tubing can be used, which may ensure that even at a lower flow rate SWRs can be maintained in hippocampal slices, similarly to that reported earlier.

Thank you for the suggestion! I agree that the tubing length in my set up could be shortened if I removed the pulsation dampener. Unfortunately the peristaltic pump that I have access to only has four rollers, and I noticed a substantial pulsation with even moderate flow rates – thus my incorporation of the pulsation dampener, inspired by Shi et al., 1990. Based on your suggestion, I added discussion of this limitation to the text to emphasize that this protocol can be improved with the use a peristaltic pump with a larger number of rollers, to preclude the use of a pulse dampener and ensure that tubing length is as short as possible (lines 670 - 679).

6) MOhm should be used instead of mOhm.

This line has been corrected to MOhm (line 427).

In summary, it has to be unequivocally demonstrated that HEC slices cut with an angle are better to study SWR properties than e.g. horizontal hippocampal slices, and the recording apparatus has to be optimized to reduce fast flow rates for maintaining oscillatory activity in dual-surface superfusion chamber.

Reviewer #2:
Alexander C. Whitebirch: An optimized acute brain slicing method for investigation of spontaneous hippocampal network activity

Manuscript Summary:
The author describes a modified slice preparation protocol, which allows investigating cellular mechanisms of hippocampal network activity (the sharp wave/ripple, in particular) in acute slices from mouse hippocampus. The manuscript is organized and written very well, illustrations are appealing and informative; all procedural steps are explained well and in sufficient detail. The protocol description will be very useful for labs intending to enter the field of hippocampal network oscillations.

(main comments)

I have one major conceptual concern: the author mentions (and I highly value his openness not to conceal this fact) that just one or two slices per hippocampus regularly express spontaneous network activity (Discussion). Why is that? Based on my experience and that of other labs working in the field (see references below), simpler approaches (i.e. without cardial perfusion) can reliably produce slices (up to five per hippocampus, depending on slice thickness) that show spontaneous network activity. The author should add some discussion to explain this issue; one possible explanation would be that his approach potentially covers more of the intermediate-to-dorsal hippocampus, while conventional 'horizontally cut' slices tend to be closer to the temporal pole…

I added a paragraph in the discussion to address this issue (lines 688 - 705). I cannot fully account for my lower yield (in terms of the proportion of slices showing spontaneous SWRs) relative to some other studies. I agree that my use of the intermediate hippocampus may indeed be a factor, as there is some evidence in the literature that spontaneous activity occurs most readily in ventral slices. I added text in the introduction and discussion to specifically address the role of the dorsal-ventral axis. Another contributing factor may be my use of slightly thicker (450 um) slices, which yields fewer slices in total. I also used adolescent and adult mice (hence the incorporation of transcardial perfusions), and the success rate may be improved with the use of younger animals.

Introduction: l. 66-67: 'For example, in vivo the hippocampus generates highly synchronous population activity, visible as oscillations in the extracellular local field potential.' - Please, add appropriate in vivo references here (e.g. point to original work by Vanderwolf, Buzsáki, McNaughton, O'Keefe, or others)!

Good suggestion - I added several relevant representative references to this portion of the introduction (line 53)!

l. 86: the author quotes examples of studies conducted in interface conditions (Ref. 4 and 6); he should not conceal that sharp wave/ripple activity, too, was investigated using this approach. In that regard, statements like 'Hippocampal slices do not typically exhibit SWRs or other forms of spontaneous network activity.' (l. 33/34) is incorrect. The author is pointed to and should give credit to earlier work (e.g. by Maier et al., J Physiol 2002 and 2003; Papatheodoropoulos & Kostopoulos, Brain Res Bull and Neurosci Lett 2002; Wu et al., J Physiol, 2002; Kubota et al., J Neurophysiol 2003; Behrens et al., Nat Neurosci 2005; Both et al., Hippocampus, 2005; Colgin et al., Neurosci Lett, 2005, and others). Please provide a more balanced overview of studies which have used acute slices to study hippocampal oscillations (that is, sharp wave/ripple- or gamma activity).

My original text was poorly written in this regard, particularly the statements like the one you identified from lines 33 & 34. The missing context was that this statement was in reference to transverse hippocampal slices of the dorsal hippocampus, maintained under submerged conditions. The abstract and introduction have been heavily revised to clarify this point. Thank you for the suggested references – I've substantially re-written the introduction to provide a more comprehensive overview regarding past work on sharp wave ripples and other network activities in slices (lines 26 – 174).

Figure 1: The author emphasizes the importance of a 12° cutting angle for preparing hippocampal/entorhinal cortex slices. Why 12°? Is there any evidence for benefits using this particular angle, or any angle at all? Since the hippocampus is a bent structure, I do not see any advantage of using a cutting angle, unless the angle is constantly adjusted with every new cut

slice; see Figure 2 in Papatheodoropoulos & Kostopoulos (2002) for illustration of angle adjustment: there, with every slice the tissue was rotated with respect to the blade of the tissue chopper; and indeed this procedure can optimize preserving the integrity of the hippocampal lamella. Please discuss this issue, and provide references for the benefits of using the 12° cutting angle!
Papatheodoropoulos and Kostopoulos (2002) Brain Res Bull 57, 187-193
https://www.sciencedirect.com/science/article/pii/S0361923001007389?via%3Dihub#FIG1

I added text to the introduction and conclusion to clarify the use of an angled horizontal slice, with specific references to several papers that recommend this slicing method (lines 123 - 135; lines 620 - 638). I didn't mean to claim that it is a superior or necessary modification to a "standard" horizontal slice, and you are certainly correct in that the "ideal" angle with respect to the hippocampal lamellae will vary along the whole dorsal-ventral curve of the hippocampus. Indeed many studies I've referenced in this protocol produce spontaneously active slices with a "true" horizontal cut. However, the angle can be implemented to preferentially create horizontal slices that parallel the transverse plane of the ventral or intermediate hippocampus. In figure 1 of the original manuscript, the brain was depicted with the ventral surface glued to the ramp and the rostral end of the brain pointed up the slope of the ramp – this "upward" angle, used by Stoop et al., 2000 and Mizunuma et al., 2014, yields more slices from the intermediate hippocampus. I added to the figure a depiction of the alternate angle, where the rostral end of the brain is pointed down the slope. With this "downward" angle, the horizontal cut will produce more slices of the ventral hippocampus. The downward angled horizontal slice is used by Jones et al., 1988, Rafiq et al., 1993, Carter et al., 2011, and ul Haq et al., 2016. I clarify this issue in the text, and explain that if one's experimental objectives do not specifically require intermediate or ventral slices, the agar ramp can be disregarded and flat horizontal slices can be used (lines 123 - 135; lines 620 - 638).

(further comments)

Abstract: uM -> µm

All instances of uM have been replaced with µm (lines 39,121,385,446,462,596,689,693) .

Protocol:
l. 117: ACSF = artificial cerebrospinal fluid (not 'cerebral')

All instances have been fixed to read "artificial cerebrospinal fluid" or "ACSF" (lines 22,38,41,69,190,584).

l.331: please correct: patch pipettes should have resistances of several megaOhms (MOhm) not milliOhms (mOhms)

This line has been corrected to MOhm (line 427).

Figure 2, and l. 353ff: Using empty syringes as pulsation dampeners is an excellent idea! The author should refer to the underlying physical (and physiological) principle of 'elastic reservoir' or 'Windkessel' effect - see https://en.wikipedia.org/wiki/Windkessel_effect for further information.
Thanks for the information! I included a mention of the Windkessel effect in the discussion, as well as a reference (lines 668 - 679).

l. 365: please indicate the approximate volume of the recording chamber!

The volume of the recording chamber is approximately 1 mL, to accommodate fluid flow underneath and above the slice. The narrowed oval shape is intended to promote laminar flow of the ACSF. The chamber design could be altered to reduce volume further, particularly if the middle layer of the chamber is excluded and the slice rests on the coverslip, as in a standard submerged recording chamber. The chamber I present here is similar in concept to the commercially available Warner RC-27LD, in which slices rest on a support layer of Lycra threads.

l. 430: 'clean' water; please specify!

The water in our lab is purified using a Milli Q system. In the text I included a brief, general description as recommended by reviewer #3: "…purified water that has been filtered to remove trace metals and other impurities" (lines 193,202,574,584).

l. 560: Strange et al. - the journal should be Nature Reviews Neuroscience.

I corrected this reference (lines 778).

Reviewer #3:
Manuscript Summary:
Alexander Whitebirch describes a protocol to obtain spontaneously active horizontal slices of the mouse brain containing the hippocampus and entorhinal cortex (HEC slices). The protocol makes use of standard methods for in vitro electrophysiology while applying a combination of optimised steps to promote cell survival and intact connectivity within the tissue. The author proposes the use of transcardial perfusion, a cutting angle, incubation of tissue under interface conditions, and a double perfusion recording chamber as important measures to obtain an improved quality of electrical activity in HEC slices, especially from adult mice. The manuscript

represents an interesting and informative description of the protocol, while showing some weaknesses in introducing the field and relevant applications and in the discussion of the applied techniques.

Major Concerns:
There are no major concerns.

Minor Concerns:
Title / Abstract:
(1) Please add "in mice" to the title. The protocol is described for use of mice and was probably only tested in mice by the author. If so, please make sure that the statements referring to this protocol only refer to mice across the manuscript.

You are correct - everything in this protocol refers to mice specifically, and I edited the title to reflect that (lines 2,3).

Introduction:
(2) The first paragraph does not efficiently summarise the general use of in vitro preparations and the citation of Karlocai (2014) is insufficient to account for them. Either rewrite or make more specific to recordings of SWR- or epileptic activity in HEC slices.

The introduction has been heavily revised to include more specific background and references regarding network activity in slices (lines 46 - 174).

(3) ACSF stands for "artificial cerebrospinal fluid". Incorrect across the rest of the manuscript.

All instances have been fixed to read “artificial cerebrospinal fluid” or “ACSF” (lines 22,38,41,69,190,584).

(4) The expression "slices" should be used consistently, not "sections".

I replaced all instances of “section” with “slice” (lines 29,38,46,137,390,688).

(5) 2nd par. and Fig. 1: The angle of 12 deg used here is not specifically cited, but rather vaguely discussed. The illustration seems inconsistent with other work cited, since placing the ventral side of the mouse brain on the block rather produces a 0 deg angle compared to the dorsal line (surface of the cortex). Please clarify!

I added a paragraph to clarify the use of an angled horizontal slice, with specific references to several papers that recommend this slicing method (lines 123 - 135; lines 620 - 638). I do recognize however that many other studies do not use an angle, and I certainly don't mean to claim that it is a superior or necessary modification to a "standard" horizontal slice. Indeed many studies I've referenced in this protocol produce spontaneously active slices with no angle. However, the angle can be implemented to preferentially create horizontal slices that parallel the transverse plane of the ventral or intermediate hippocampus. In the original figure the brain was depicted with the ventral surface glued to the ramp and the rostral end of the brain pointed up the slope of the ramp – this "upward" angle, used by Stoop et al., 2000 and Mizunuma et al., 2014, yields more slices from the intermediate hippocampus. I added to the figure a depiction of the alternate angle, where the rostral end of the brain is pointed down the slope. With this "downward" angle, the horizontal cut will produce more slices of the ventral hippocampus. The downward angled horizontal slice is used by Jones et al., 1988, Rafiq et al., 1993, Carter et al., 2011, and ul Haq et al., 2016. I clarify this issue in the text, and explain that if one's experimental objectives do not specifically require intermediate or ventral slices, the agar ramp can be disregarded and a flat horizontal slicing plane can be used (lines 123 - 135; lines 620 - 638).

(6) 3rd par.: Complex spontaneous activity in interface recording chambers has not been properly discussed and inadequately referenced. See papers from Heinemann and Draguhn labs.

Thanks for the suggestions - I revised these paragraphs to include more background on interface chamber studies and I added references to works from both U. Heinemann and A. Draguhn (lines 52 - 105, 137 - 169, 640 - 661).

(7) The difference between an interface recording chamber (Oslo-type or Haas-type) and interface storage has not been explained. Many papers, including some of the citations here, incubate in actual interface recording chambers with running perfusion and possibly recordings of the activity before the transfer. One would expect differences to the simplified interface storage chamber described here.

I agree, one would expect differences between a true interface chamber and storage of slices in a simplified interface-style holding chamber! In the introduction I clarified that the method presented in this protocol represents a "compromise" between fully submerged preparations and a true interface recording chamber with running perfusion (lines 149 - 159). I also added text to the discussion to address this limitation (lines 640 – 655, 664 - 679).

Protocol:
(8) Line 302, step 5.9.: Transfer of slices is not explained. How and using which tools?

Slices are transferred one at a time from the microtome chamber to the interface-style holding chamber using a plastic transfer pipette (line 390). I added this transfer pipette to the list of materials.

(9) Line 323, step 6.2.: Same as (8)

I added text to the protocol to explain this point. The slices are transferred with the use of fine forceps. The experimenter holds a corner of the lens paper the slice is resting on – the slice will stick to the lens paper. Using the forceps, place the lens paper and slice into the recording chamber with the slice facing down. In this way, the lens paper can then be "peeled" away, leaving the slice submerged in the recording chamber (lines 432 – 436).

(10) Basic steps to record LFP signals are not explained (Using multiclamp amplifier listed in materials?). Some components for whole-cell recordings are mentioned, but no whole-cell recording is shown and is does not really seem to be part of this protocol. I suggest removing whole-cell recording components and solutions from the manuscript.

I added additional detail to the protocol regarding the LFP recording steps (lines 402 – 482). I did also follow your suggestion and remove all whole-cell recording portions from the manuscript.

Figures and Tables:
(11) Table 1: Define "clean water", term seems uncommon compared to aqua bidest, deionized water etc. Maybe something similar to citation 13: "Solutions should be made up in purified water that is free of trace metals and other impurities."

I replaced the term "clean water" with a more specific description (lines 193,202,574,584).

(12) Table 3, line 450: pH value is brought UP from an acidic pH (low value) to a more neutral pH (~7.2) with KOH.

I removed the parts of the protocol concerning intracellular recordings.

Discussion
(13) Line 460: 32 deg Cel. does not represent a physiological temperature for mice (~37 deg C). These recording temperatures (32 - 34 deg C) are simply established as a compromise between sufficiently high activity and stability of slice preparations.

This phrase has been removed.

(14) Line 504: Has the NMDG protocol been tested to successfully produce spontaneously active HEC slices from wild-type mice? Can you safely claim an improved quality in terms of this protocol?

I have not used the NMDG protocol, so indeed I cannot claim from personal experience that it would be in improvement with specific regards to this protocol. This sentence has been removed.

(15) Discussion of the protocol seems narrow. Many papers produced comparable oscillations (judging by Fig. 3) without transcardial perfusion; although this was shown to be very beneficial for slices form adult animals, the perfusion poses a risk to animal welfare and requires ethical approval and training. Experiments without imaging could possibly be performed without transcardial perfusion.

I expanded upon the discussion to include more in the way of alternatives and limitations to this protocol (lines 593 – 705). The use of transcardial perfusions was included here to provide detailed steps for researchers using adult or aging animals, however you are correct in that perfusions are not strictly necessary and shouldn't be performed unless necessary. I added text to address this issue in the discussion (lines 602 - 613).

(16) The low number of active slices makes me wonder, although maybe only one or two slices from each hemisphere could be of sufficient quality for a specific experiment. This definition is at the same time imprecise, as the quality criteria for SWR activity should be chosen to be sufficient for the individual experiment while maximising the use of tissue.

I agree that the number of active slices required will depend on one's specific experimental objectives. In this case, my definition for an "active slice" was one in which multiple spontaneous SWRs were observed in CA2/CA3 or CA1. I cannot fully account for the low number of active slices produced by this protocol, but I added a paragraph to the discussion to address possible factors (lines 688 - 702).

Material and solutions
(17) whole-cell patch clamp-specific solutions seem irrelevant for the protocol.

I removed the parts of the protocol concerning intracellular recordings.

(18) It is mentioned that the storage chamber and recording chamber was custom-built using a 3D-printer. The model and the material used for printing is missing from the Material/Equipment list. Maybe consider providing model-files?

I added both the 3D printer and the material used to the Materials/Equipment list. The holding chamber is a basic acute slice chamber designed by ChiaMing Lee and the file can be found on the NIH 3D print exchange at https://3dprint.nih.gov/discover/3dpx-001623 (materials list). I designed the recording chamber and included the model file in this revision (materials list).

Reviewer #4:

Manuscript Summary:

The author describes techniques for preparation of hippocampal slices; many of the details of the described techniques are indeed very useful for obtaining healthy slices.

Major Concerns:

However, the text is vaguely written, and should be edited for precision and clarity (below are just few examples out of many...). As of now, it reads more like a memo for the author or people working in the same lab and already familiar with the techniques. I am not sure that unexperienced person, say undergraduate student starting to work in the lab would be able to reproduce these procedures in a running lab; or, for that matter, a postdoc without prior experience would be able to start a lab using this description.

That said, the paper does contain useful hints and detail.

Thank you for your feedback – I've added detail to much of the protocol (lines 176 – 482), and addressed below the specific points you raised.

Minor Concerns:

Tables 1-3 and preparation of solutions

- Table 1 and 2 legend is mixed up

I reordered the legends to reflect the table files (lines 574 – 590).

- please fit 5 columns of each table in one page

- as of now, it reads as if CaCl2 and MgCl2 have MW of 1

- why not to make stock solutions also for NaCl, KCl, NaHPO4; also NaHCO3?

I adjusted the width of the columns so that all five columns fit onto one page. In order to distinguish between the CaCl2 and MgCl2 solutions and the other chemicals, the tables include a second row of labels, with the third column labeled as "stock concentration (M)". There is no specific reason why stock solutions couldn't be made for the other chemicals you mention – the lab I am in just happens to purchase stock solutions of CaCl2 and MgCl2, so the tables are written to reflect the way that I prepare sucrose and ACSF. Certainly one could prepare stock solutions of other components as well.

- what is the point of keeping stock of NaCl solution at -20C° ???

I rewrote that portion of the protocol, NaCl solution does not need to be stored at -20C (lines 192 - 194).

- since the composition of intracellular solution is provided, please consider showing an example recording.

I removed the parts of the protocol concerning intracellular recordings.

- Table of materials - please make font size uniform (unless different font size is intentional, then explain what it supposed to stress)

All text in the table of materials has been made size 12 font.

- "Clean water" - ??? do you mean distilled? Tab water is also 'clean'.

I updated the text to be more specific, indicating that solutions should be made with "purified water that has been filtered to remove trace metals and other impurities" (lines 193,202,574,584).

- "To visualized recorded cells... " -> "To label recorded cells for morphological reconstruction..."

I removed the parts of the protocol concerning intracellular recordings.

- "... single-cell
53 recordings for which a glass pipette must be stably affixed to the cell membrane. " - this is a weird description of an intracellular recording...

I re-wrote this portion of the introduction and removed this sentence (lines 46 – 50).

- mold for agar preparation - petri dish?

A petri dish would work, but I've been using a rectangular plastic container that once held glass capillaries. The rectangular container is approximately 4 cm by 4 cm by 12 cm. When filled with agar dissolved in purified water, I tilt the container along its long axis so that when the agar solidifies it forms a ramp 12 cm in width. For each experiment I cut out a small ramp, roughly 2 cm in width. I added some more detail on this point to the protocol (lines 201 - 209).

- agar ramp - please give approximate dimensions

The ramp should be approximately 4 cm along the angled surface, 0.8 cm in height, and 2 cm in width. I added dimensions to the protocol (lines 253 - 256).

- what for is carbogen line in petri dishes? If petri dish is filled to ~3-5 mm depth, carbogen might go straight in the air rather than diffuse sideways. I would rather keep chilled solution bubbling in a beaker, and pour it into the dish just before use.

You are correct - in fact, I don't fill the petri dishes with sucrose solution until the last moment before beginning the perfusion and dissection. I re-wrote this portion of the protocol to clarify and emphasize this point (lines 297 – 299). The carbogen lines are directed into each petri dish to ensure that carbogen is bubbling continually through the sucrose in all stages of the protocol, but I believe you are correct in that the more important factor is that carbogen is bubbled though the sucrose stored in a beaker before the dissection begins.

Fig. 2B - where and how ACSF is supplied to/removed from the chamber?

The ACSF is supplied by two tubing inlets at one end of the oval chamber, and an outlet attached to a vacuum is positioned at the other end of the chamber to remove ACSF. I added text to the figure legend and updated the figure to include this (figure 2).

Fig. 3A - labeling EC and DG might be useful.

I added labels for the DG and the EC (figure 3).

Fig. 3B - How stimulation intensity was adjusted to compare fEPSPs?

I stimulated each slice with a range of stimulus intensities. Typically maximum responses are observed with a stimulus intensity of around 64V, so the representative fEPSPs are averaged responses to 64V stimulation (figure 3).

Fig. 3C - what is the rationale for showing recordings at different scales and differently filtered? Comparison might be easier if scale and filtering are same...

These two traces are filtered and scaled differently because they are presenting the two different aspects of the SWR signal: the top trace is filtered to isolate the high frequency oscillation present in the SP layer, and the bottom trace is filtered to emphasize the low frequency sharp wave in the SR layer (figure 3).

Reviewer #5:

Manuscript Summary:

This protocols aims to provide the optimal conditions for preserving the hippocampal network integrity in order to recapitulate the endogenous sharp wave-ripple activity in vitro. For this purpose author recovers the slices in an interface storage with fast ACSF flow rate and records the activity from a submerged chamber as Maier and colleagues suggested. (Maier et. al., 2009). In addition, thick (450um) slices were cut at a 12-degree angle to maintain the connections between hippocampus and entorhinal cortex. Author claims when the indicated steps were followed one would obtain active and healthy slices presenting large evoked field responses and spontaneous sharp-wave ripples.

Major Concerns:

It is unclear why one has to adapt the cut with the 12 degree. In referred articles (Maier et. al., 2009-PLos ONE; Schlingloff et.al., 2014-j Neuosci) the spontaneous SWR activity was successfully detected in high yield (90%) when the regular horizontal cutting was applied where as in this protocol one can obtain only one or two active slices. And there isn't any comparison of the quality of the recordings.

In the original manuscript I was not clear in my rationale for including the angled slice approach. I didn't mean to claim that it is a superior or necessary modification to a "standard" horizontal slice, and you are certainly correct in that this angle is not necessary for the primary objective of producing slices exhibiting spontaneous SWR activity. Indeed many studies I've referenced in this protocol produce spontaneously active slices with no angle. I added a paragraph to clarify the use of an angled horizontal slice, with specific references both to studies that recommend this slicing method as well as studies that use a true "flat" horizontal slice (lines 123 - 135, 620 - 638). My rationale in including this aspect to the protocol is that the angle can be implemented to preferentially create horizontal slices that parallel the transverse plane of the ventral or intermediate hippocampus. In the original figure the brain was depicted with the ventral surface glued to the ramp and the rostral end of the brain pointed up the slope of the ramp – this "upward" angle, used by Stoop et al., 2000 and Mizunuma et al., 2014, yields more slices from the intermediate hippocampus. I added to the figure a depiction of the alternate angle, where the rostral end of the brain is pointed down the slope (figure 1). With this "downward" angle, the horizontal cut will produce more slices of the ventral hippocampus. The downward angled horizontal slice is used by Jones et al., 1988, Rafiq et al., 1993, Carter et al., 2011, and ul Haq et al., 2016. I cannot account for my lower yield (in terms of the proportion of slices showing spontaneous SWRs) relative to some other studies. Contributing factors may be my use of slightly thicker (450 um) slices, which yields fewer slices in total, and my use of the intermediate hippocampus, which may produce spontaneous activity less readily than more ventral tissue. I also used adolescent and adult mice (hence the incorporation of

transcardial perfusions). I added text to the discussion section to address this issue (lines 688 - 702).

3D print file for chamber

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Supplemental Coding Files

dual surface chamber_Whitebirch_07.29.20.stl