Title: Perforated Multi-Electrode Array recording in hypothalamic brain slices

Running head: Perforated Multi-Electrode Array recording

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i. Abstract

The ability to record ensemble action potential (AP) discharge frequencies from large populations of neurons over extended periods of time *in vitro* offers clear advantages in neuroscience and circadian biology research. Here, we provide an overview of a step-by-step method to perform multi-site extracellular AP activity recordings in suprachiasmatic and medio-basal hypothalamic nuclei brain slices, using a state-of-the-art perforated Multi-Electrode Array system. Further, we describe in detail a setup architecture which systematically delivers stable, high-quality recordings with excellent anatomical accuracy and consistency. We also provide some procedural, technical and methodological troubleshooting notes and examples of good quality recordings.

ii. Keywords: Perforated Multi-Electrode Array, electrophysiology, suprachiasmatic nuclei, medio-basal hypothalamus, multi-unit activity, circadian

1. Introduction

The development of multi-electrode methods to simultaneously record and analyze ensemble activity from circuit-wide neuronal populations represents a powerful advance in neuroscience (1,2). This is especially useful in circadian biology studies as these tools can simultaneously probe the unfolding landscape of activity-dynamics of large populations of neurons with incredible temporal and spatial resolution (3-9). However, acute brain slice recording performed with traditional Multi-Electrode Array (MEA) systems can suffer from a number of shortcomings, such as poor signal-to-noise ratio (due to poor contact between the brain slice and recording electrodes) and unintended tissue movement over time. These become particularly apparent when measuring electrical output from slices over several hours or at the circadian time-scale. From our own experience and through the work of others (e.g. (3,4,10)) we have concluded that these and other technical issues are readily resolved by using dual-perfusion perforated MEA (pMEA) systems. For example, to make multi-site recordings, the surface of the brain slice must be in continuous contact with the fabric of the array, and in conventional MEAs this can restrict oxygenation and nutrient supply to the recording surface and compromise slice viability. The pMEAs solve this important issue by allowing artificial cerebrospinal fluid (aCSF) to penetrate deep into the parenchyma, providing ample oxygenation and nutrient supply to the brain slices (11). Together, these highlight how the pMEA systems are especially powerful for recording electrical activity in brain slices over several hours. In this chapter, we therefore document an overall step-by-step

method of how to achieve such recordings, placing tissue survivability and recording stability at the forefront while attaining excellent signal-to-noise ratio.

2. Materials

2.1 Equipment and background

2.1.1 MEA2100-HS2x60-System workstation

We describe the setting up and specification for perforated MEA recording using the new state-of-the-art dual-MEA2100-HS2x60 head-stage system from Multi-Channel Systems (MCS GmbH, Reutlingen, Germany; *see* Figs. 1-2 for our setup layout; and http://www.multichannelsystems.com/systems/multiwell-mea2100-2x60-system). This device integrates data acquisition from 120 MEA recording electrodes (60 electrodes per well/chamber/probe: hereafter appropriately referred to as pMEA probe/chamber/array), and communicates with the computer via the MEA2100-interface board. In addition, a 2-channel temperature controller (TC02), two heatable perfusion cannulas with temperature sensor (PH01), 2 peristaltic perfusion systems (PPS2), a Constant Vacuum Pump (CVP) and other accessories are needed (detailed below and shown in Figs. 1-2). We recommend purchasing all these from MCS, if possible, as they have been designed for the optimal functioning of the MEA system. The methods and procedures we describe below can be easily adapted to use with the single head-stage perforated MEA2100-HS60 or with the older MEA1060 amplifier systems (*see* MCS website). The dual head-stage system (labelled as 1 and 2 in Figs. 1d and 2d), however, offers the unique advantage for two recordings to be performed in parallel from brain slices collected from two independent animals under appropriate experimental conditions (e.g. knock-out *vs* its congenic wild-type littermate; treated *vs* control animal). This provides an ideal internal control. For an experienced user, setting up for dual-pMEA recordings, including aCSF and brain slice preparation and acclimatization, takes approximately 3-4 hours.

2.1.2 Perforated MEAs (60pMEA100/30iR-Ti-gr or 60pMEA200/30iR-Ti-gr)

The 60pMEA100/30iR-Ti-gr and 60pMEA200/30iR-Ti-gr are pMEAs with 60 titanium nitride electrodes, 30 µm in diameter each, embedded in a perforated polyimidine foil (Figs. 4a and 5a). The perforations allow aCSF exchange from above and below the pMEA probes. When carefully setup, this permits the formation of a negative pressure/suction underneath the brain slice (see below). The electrodes in the 60pMEA100/30iR-Ti-gr MEAs are 100 µm apart arranged in a 6x10 layout (ideally suited for small brain structures such as the

suprachiasmatic nuclei (SCN)), while in the 60pMEA200/30iR-Ti-gr the electrodes are 200 µm apart arranged in an 8x8 layout (suited for larger brain circuits such as the medio-basal hypothalamus (MBH); see Figs. 4a and 5a). Unless necessary, we recommend to use pMEAs with 6mm high glass ring (identified by the code -Ti-gr), rather than plastic rings (-Ti-pr), as these are more durable, and with due diligence and care can be used for at least 40 experiments. From new and between use, store pMEAs overnight at 4°C in an enzyme-active powder detergent (such as 1% Tergazyme; Sigma-Aldrich).

2.2 Solutions

Prepare incubation/cutting and recording aCSF solutions in ultrapure deionized and distilled water (For example, using the MilliQ water purifying system to attain a sensitivity of ≤18 MΩ-cm at 25^0C; Merck Millipore). When preparing these aCSF solutions use analytical grade salts (For example, VWR-AnalaR NORMAPUR grades). For accuracy, use calibrated volumetric flasks to prepare and/or dilute all solutions (For example, Fisherbrand™ clear borosilicate glass with Stopper). Makeup 10x and 5x stock solutions for recording and incubation/cutting aCSF solutions, respectively. It is advisable to store these solutions at 4^0C. Dilute the incubation solution from the 5x stock on the day before recording and keep overnight at 4°C. Prepare the recording solution from the 10x stock on the day of recording.

2.2.1 Cutting solution

The composition of the cutting/incubation aCSF can slightly vary across laboratories, but ours constitutes in mM: NaCl 95; KCl 1.8; KH_2PO_4 1.2; $CaCl_2$ 0.5; $MgSO_4$ 7; $NaHCO_3$ 26; glucose 15; sucrose 50; Phenol Red 0.005mg/L; oxygenated with 95% O_2; 5% CO_2; pH 7.4. We recommend using such low Na^+/Ca^{2+}, high Mg^{2+} sucrose-based cutting/incubation aCSF while preparing SCN and MBH brain slices as this maintains and protects the tissue from unavoidable anoxia and neurotoxic cell death that occurs during decapitation, dissection, and slicing (12).

2.2.2 Recording solution

The composition of the recording solution is identical to the incubation/cutting solution, except for the following in mM: NaCl 127; $CaCl_2$ 2.4; $MgSO_4$ 1.3; sucrose 0. It is advisable that for long-term recordings, antibiotic solution (such as Gentamicin, Sigma G1272 1mL/L) is added to the aCSF. Set the water bath at 1°C higher than the temperature setting chosen for

the heatable perfusion cannulas (PH01, *see* below), and warm the aCSF solution in the water bath for at least 1 hour. This allows the aCSF solution to uniformly reach the desirable temperature before gassing. Gas the aCSF to saturation, which normally takes ~20 minutes, before adding $CaCl_2$. We use a sealed aCSF reservoir-flow system to store and circulate aCSF (*see* **Note 1** and Figs. 1a and 2).

Although the osmolarity of the aCSF solutions can be estimated/calculated using empirical methods, we strongly recommend to measure this directly with an osmometer prior to use. For both solutions, this should be in the 305-315 mOsmol/kg range. Addition of the pH indicator Phenol Red in the aCSF provides an instantaneous visual cue (colour change) to any alteration in the gaseous status/content of the aCSF. This is helpful for monitoring aCSF gas saturation both during gassing and recording.

3. Methods

3.1 Brain slice preparation

Deeply anaesthetize the animal with isoflurane (Baxter, Norfolk, UK; code: FAGG9623) to minimize pain, discomfort and stress, and kill by cervical dislocation followed by decapitation. Using a razor blade, make an incision along the scalp, carefully cut away the top and side parts of the cranium and remove the brain. During this process avoid stretching or applying mechanical strain to the optic nerve since these can damage the optic chiasm and pre-optic regions of the hypothalamus, including the SCN. Rest the brain (ventral side up) on an aCSF-soaked tissue paper and prepare a block of tissue containing the whole hypothalamus by grossly cutting away the anterior and posterior aspects of the brain. Glue the caudal side of the tissue block (rostral side facing uppermost) on the stage of a vibroslicer. Once in the cutting chamber, submerge the tissue block in ice-cold incubation/cutting aCSF solution and, with the ventral side of the hypothalamic block facing the blade, carefully cut coronal hypothalamic slices containing the SCN and MBH. As a rule-of-thumb, we recommend using ceramic blades as these are sharper than stainless steel ones, producing less damage to the tissue. The section thickness should not be less than 200 µm (250 µm is an ideal thickness but you may want to cut at 300-400 µm for long-term recordings: *see* **Note 11**). Transfer the slices to a holding chamber in which the incubation/cutting aCSF solution is continuously gassed at room temperature. Allow the slices to rest and recover here for at least 30 min. Finally, transfer the slices to a separate

holding chamber containing continuously gassed recording aCSF solution, and leave the slices to acclimatize here at room temperature for at least another 30 min for full recovery before transferring to the pMEA chamber. Use these time windows to setup the MEA system.

3.2 MEA setup: Overview for a one-off setup from new

We provide a comprehensive set of diagrams and photographs to show the overall setup layout of our dual-head-stage pMEA system (Figs. 1, 2 and 3), which offers efficient, ergonomic and easy access during slice setup. This way of assembly also enables exceedingly stable and high-quality recordings to be performed from brain slices in a systematic way, allowing great anatomical accuracy and consistency across experiments. The two head-stages (labelled as 1 and 2 in Fig. 1d and 2d, and built into one unit) and pMEA chambers operate independently, permitting independent aCSF/drug delivery to the recording tissues and acquisition. It is advisable to assemble the whole unit, except for the aCSF reservoirs and water bath, in a Faraday cage to shield from electric fields and electromagnetic radiation that can contribute noise to the recordings.

For each of the pMEA chambers there are two perfusion systems; an “upper” and “lower” perfusion (Figs. 1-2).

3.2.1 Upper perfusion system

The upper perfusion supplies the brain slices with a continuous “inflow” of fresh oxygenated aCSF and a “suction outflow” to remove excess aCSF waste. To drive the inflow and outflow of each pMEA chamber we use, and recommend using, two separate peristaltic perfusion systems (MCS; PPS2). Each PPS2 contains two independent pumps. Use one for inflow and the other for outflow (Figs. 1-2). The aCSF solution is guided into the pMEA chamber by a cannula bearing an inline heating system (PH01). Position the inflow and outflow cannulas as shown in Figs. 1d, 1*d1* and 3j-k to minimise turbulence during solution exchange and maximise drug accessibility to the slices.

The components are:

Inflow, in sequence;

- Two 2L bottles filled with aCSF (place these in a water bath. Use larger aCSF reservoirs for recordings over 8 hours. Estimate the volume needed by your flow rate; Figs. 1a and 2a).
- Two ramps of stopcocks Luer-lock (e.g. 4 one-way stopcocks with a 3-way stopcock at one end; Vygon Ltd, REF873.15) to hold and vent the drug reservoirs (can be fitted with plastic barrels from 20 mL syringes: Figs. 1b, 2b and 2l).
- Two hypodermic needle shafts or small metal barrels (*see* **Note 2** and Fig. 1*a4*).
- Two PPS2 peristaltic pumps.
- Two PH01 temperature units (connect these to a TC02 temperature controller) with cannulas and magnetic perfusion holders (MPH).
- Tubing (2x ~1.6 mm internal diameter; MCS code: PPS2-Set-F).
- Connectors for tubing assembling (MCS code: PPS2-Set-F).

Outflow, in sequence;

- Two metal cannulas/suction tubes with beveled end (e.g. Warner Instruments, ST-1L/R 64-1401: Fig. 3*k3*).
- Two magnetic perfusion holders (MPH).
- Tubing (2x ~1.6 mm internal diameter; MCS code: PPS2-Set-F).
- Large waste bottle (store on the floor).

Other accessories

- A binocular microscope.
- Halogen cold light source fitted with dual-gooseneck light guards. If you have a dual head-stage MEA system, using two of these is advisable to provide illumination from above and underneath the pMEA chambers. The light sources are important for MEA setup, tissue placement, and *in situ* photographing of tissue-pMEA chamber assembly (*see* Figs. 2, 4 and 5).
- Tissue anchor (e.g. Warner Instruments; code 64-1421: *see* Fig. 3*k1*).

3.2.2 Lower perfusion system

The lower perfusion is partly driven by gravity and partly by the CVP pump (Fig. 1-2). Its main purpose is to provide continuous gentle suction underneath the pMEA chamber using

fresh and oxygenated aCSF. This draws the slices firmly onto the electrode contacts at the bottom of the pMEA chamber, providing several benefits (*see* introduction and Fig. 1*d1*).

The components are:

Inflow, in sequence;

- The two aCSF-filled 2L bottles mentioned above (if running two pMEA chambers set these so that one bottle supplies fresh aCSF to the upper and the other bottle to the lower perfusion systems, as shown in Figs. 1-2).
- Two 3-way stopcocks Luer-lock taps (e.g. Kendall 170030; *see* also Fig. 1 labelled as Tap-A and -B).
- Two 20 ml syringes (*see* Tap configuration key in Fig.1).
- Two perfusion elements (MEA2100-PE2x60: Figs. 1*d1* and 3a).
- Two rubber O-rings (o-Ring-PGP: Figs. 1*d1* and 3*b1*).
- Tubing (2x ~1.6 mm internal diameter; MCS code: PPS2-Set-F).
- Connectors for tube assembling (MCS code: PPS2-Set-F).

Outflow, in sequence;

- Tubing (2x ~1.6 mm internal diameter; MCS code: PPS2-Set-F).
- Two 3-way stopcocks Luer-lock taps (as above, labelled as Tap-C and -D).
- Two 18G hypodermic needles (file-down the sharp beveled end, and attach to one end of the 3-way tap (*see* Fig. 1 and **Note 3**).
- A Y-tubing connector (*see* Fig. 1).
- A Constant Vacuum Pump (CVP, *see* Figs. 1 and 2), and appropriate tubing (>2 mm internal diameter) and connectors.

3.3 MEA recording setup

To protect the MEA system and to prime the tubing pipework initiate the MEA setup using distilled water. Ensure that the system is running smoothly before switching to aCSF. The overall aim is to run the upper and lower perfusion systems to establish a smooth solution exchange in the pMEA chambers. To achieve near noise-free, high-quality recordings with lasting stability and durability, three crucial conditions must be attained: ***i)*** the pMEA chambers must be tightly sealed onto the blue rubber O-ring and perfusion elements

(perfusion elements, MEA2100-PE2x60, are part of the baseplate); ***ii)*** there must be **NO** air bubbles in any part of the tubing and within the water pool that is contained by the rubber O-ring and sandwiched between the underside of the pMEA chambers and the perfusion elements; and ***iii)*** the water pool underneath the pMEA probe is well-contained by the rubber O-ring and has not overspill during setup (regions outside the rubber O-ring **MUST BE** completely **DRY**; *see* **Note 4** and *see* Figs. 1*d1* and 3).

3.3.1 Priming the perfusion and vacuum tubing

Prepare the lower perfusion first. Addition of a binocular microscope (not shown in the diagram, but present in the photograph in Fig. 2) is strongly advised as this allows the setup process to be supervised to greater precision as well as permitting more accurate brain slice positioning and subsequent image capture.

Inflow of the lower perfusion

- Immerse the ends of the four perfusion tubes (two for upper and lower perfusion inflows, respectively) into a 2L bottle filled with distilled water (use a third and separate bottle designated to circulate water through the system only). Ensure that the tubes are fully immersed with their ends resting at the bottom of the bottle.
- Open the lid of the MEA (*see* **Note 5**) to expose the perfusion elements (MEA2100-PE2x60).
- Thoroughly dry two blue rubber O-rings and place each one snuggly into the grove/notch of the perfusion elements (*see* Figs. 1*d1*, 3b and 3c, and **Note 6**).
- Fit the Taps (A to D) with 20 mL syringes (*see* Figs. 1-2).
- Start setting up the perfusion element of head-stage 1, then move to perfusion element of head-stage 2 (Fig. 1), if you plan to setup and run two pMEA probes in parallel.
- Place Tap-A in Position-A and half-fill the attached syringe with water by a gentle pull on the plunger.
- Place Tap-A in Position-B and gently push the plunger until a water column starts to appear at the inlet of the perfusion element, forming a concave meniscus (*see* Fig. 3c).
- Using Tap-B perform similar preparation to the second perfusion element if you plan to record from two pMEA probes in parallel.

Outflow of the lower perfusion

- Half-fill the syringe attached to Tap-D with distilled water (notice that Tap-D is on the same perfusion system as Tap-A, but attached to the outlet tubing connected to the CVP).
- Place Tap-D in Position-A and gently push the plunger until a water column starts to appear at the outlet of the perfusion element, forming a concave meniscus (Fig. 3c).
- Continue to gently push water through until the two water pools merge, engulfing both the inlet and outlet of the perfusion element. It is **IMPORTANT** that the surface tension of the water pool is maintained and that the area immediate to the inside region of blue rubber O-ring is kept dry (Fig. 3c).
- Using Tap-C perform similar preparation to the second perfusion element.
- During setup simply use your index finger to "flick" at the tubing and connectors to remove trapped air bubbles.

3.3.2 pMEA placement on MEA perfusion element

- After a careful and thorough rinse with distilled water (*see* **Note 7**), meticulously dry the electrode contact pads (Fig. 3*e1*) and underside of the pMEA chamber with soft tissue paper. **AVOID** any contact with the area of the pMEA that bears the perforations.
- Follow the guidance of MCS for where the pMEA reference electrode should be pointing. For example, for the 6x10 and 8x8 layouts, the reference electrode should point to the left, aligning with electrodes 14 and 15, respectively (*see* Figs. 3-5). Once the reference electrode is identified and correctly aligned, carefully lay-down the pMEA onto the blue rubber O-ring by doing the following (*see* Fig. 3d-e; we call this the "cover-slipping-like" lay-down as the action to accomplish this resembles microscope slides cover slipping): Place one edge of the pMEA chamber onto the rubber O-ring and keep it in place with two fingers while gently lowering the opposite end of the chamber toward the water pool at the center of the perfusion element (Figs. 3c-d). Once the underside of the pMEA chamber touches the water pool, keep lowering the unit in a "cover-slipping-like" fashion until it forms a water-tight seal onto the blue rubber O-ring (*see* Fig. 3e). Do this gently, slowly and gradually until a perfect seal is achieved. Before completing the seal sufficient water should be added (use the syringes). The aim is to have the entire volume encased between the underside of the pMEA chamber and the perfusion element completely filled, leaving

no gaps or air bubbles once the pMEA is sealed onto the rubber O-ring (compare Figs. 3e-f). This is a critical step and tricky to get right, requiring diligence and dexterity to avoid overspills and/or introducing air bubbles. The appropriate volume of water (Fig. 3c) needed to do this comes with practice. Use the syringes to add extra or remove excess water during pMEA placement, but be careful not to introduce air bubbles or to cause an overspill outside the confinement of the blue rubber O-ring.

- Repeat the above to prep the second pMEA chamber.
- Finally leave Tap-C and -D in Position-B to close the under flow system.
- Inspect the assembly. **IMPORTANT**: make sure that all the water is contained within the confinement of the blue rubber O-ring and that there is no overspill. Make sure that the electrical contact pads on the pMEA are dry and debris-free.
- Slowly close and lock the MEA. At this stage two sheets of parafilm, one for each pMEA probe, can be added. These guard the interior of the MEA and gold connector pins against potential pMEA chamber floods which if happen during the setup and/or recording process can cause acute and long-term issues, such as severe damage to the equipment. This is therefore strongly advised, and if you wish to do this, *see* **Note 8** and Fig. 3g-i.
- Use the two syringes attached to Tap-C and -D to fill the tube connected to the CVP.
- Place Tap-C and -D in Position-A to maintain the water column in the CVP tube in place.
- Open Tap-A and -B by placing them in Position-C, to slowly begin filling the pMEA chambers with distilled water by gravity (*see* **Note 9**). Once the base of the pMEA chambers is water-coated (supervise this under the binocular microscope), close the inflow by returning these Taps to Position-B.
- The inflows for the lower perfusion systems are set to run very slowly (~0.8 mL/min).

Inflow and outflow of the upper perfusion

- Setup the inflow and outflow for the over perfusion system as shown in Figs. 1, 2 and 3, using the magnetic perfusion holders provided (*see* also **Note 10**).
- Place the inflow cannulas as shown in Figs. 1d, 1*d1* and 3j-k. This minimizes the mechanical strain on the brain slice produced by the incoming inflow solution currents.

- Place the outflow cannulas as shown in Figs. 1d, 1*d1* and 3j-k. It is best if the tip of the cannulas is beveled. This ensures continuous skimming of the aCSF solution surface without any intermittent make-break contact with the solution, which can introduce electrical noise and/or cause turbulence in the aCSF-filled chamber that supports the tissue.
- Switch on the PPS2 peristaltic pumps. For optimum stability during slice recording set the inflow to 1.8 mL/min and outflow at 17 mL/min.
- Turn on the CVP (usually set at 40 to 43 mbar, depending on slice thickness and brain region, *see* **Note 11**).
- Open the taps (Tap-A to -D in Position-C) and turn on the peristaltic pumps to start running the over and under perfusion systems. Allow water to circulate for at least 10 min.
- Inspect the system making sure that all is running smoothly, then switch to aCSF.
- Run with aCSF for at least 20 min to ensure complete aCSF/water exchange.
- Turn on the MEA head-stage, heater controller and MCS software (*see* below). **IMPORTANT**: Do not turn on the MEA head-stage while running with water. To do so may reduce the life of the pMEA electrodes.
- Run the MCS acquisition software, making sure that the hardware baseline is stable and within acceptable noise level (~ ±5 µV, except reference electrode number 14 or 15 which should be considerable less; ~ ±1 µV).
- Leave the software to run while you place and position the brain sections in the pMEA chambers.

3.3.3 Positioning and aligning the brain slice onto the recording electrodes

- Momentarily stop the under perfusion by closing Tap-A to -D (Position-B for Tap-A and -B, and Position-A for Tap-C and -D).
- Cut off the tip of a 3 mL disposable plastic Pasteur pipette (for example Sigma, Z331740-1PAK) to create an opening with a wider aperture (~ 8mm in diameter). Carefully draw the required brain section into this modified pipette by using suction, then, transfer the section into one of the chambers. Avoid using a paintbrush to do this as the bristles could damage the slice. The Pasteur pipette transfer method also ensures that the slice is always maintained in a pool of gassed aCSF.

- Allow the slice to rest flat at the bottom of the pMEA chamber, then with a pair of fine forceps gently place a slice anchor over the tissue (*See* Fig. 3*k3*).
- Using a paintbrush with very fine natural bristles and under the visual guide of the binocular microscope, maneuver the anchored slice until the brain region you want to record from lies directly above and is appropriately aligned with the electrodes.
- If you are running two pMEA probes, repeat the above for the second chamber.
- Leave the slices to settle for a few minutes.
- Slowly open Tap-C (Position-C). This exposes the slice to the gentle negative suction produced by the CVP. While opening the tap you should therefore observe the slice being drawn down towards and onto the electrode array. Within a few minutes extracellular AP activity should start appearing on the computer screen (*see* Figs. 4-5). If you do not see any of these consult **Note 13**.
- Repeat this for the second pMEA chamber by opening Tap-D (Position-C).
- Except for your reference electrode, your noise level should now be at ~ ±8-10 µV.
- Then, carefully open Tap-A and -B (Position-C) to allow the CVP to draw some of the aCSF directly from the underflow inlet/aCSF reservoir.
- If all is well, the recording should now be stable and there should be a gradual increase in AP discharge frequency as the slices stabilize and settle on the electrodes.
- Leave the slices to acclimatize for at least 30 minutes before starting to record the data. If you choose to start saving the data straight away, do not use the first 30 minutes of the data in analysis as this typically represents instability in recordings.
- It is advisable to always capture an image of all recorded slices at an appropriate magnification *in situ* (e.g. Figs. 4-5). For this a high-definition webcam is sufficient. Take a photograph of the electrode array with the same magnification. This can be done prior to tissue placement. You can then align and superimpose the two images to visualize the precise location of each recording electrodes, if they are not already visible through the brain slice (*see* Figs. 4-5). **Tips**: Use anatomical landmarks in the slice, such as ventricular space, tissue edges, fiber bundles, etc., to consistently align and position the recording region of interest with a predetermined electrode (*see* Fig. 4-5). This will approximately maintain anatomical consistency across your recordings which may become important during analysis. Further, try to perform your recordings in slices cut at roughly the same level on the rostral-caudal axis.

4. Software, data preparation and analysis

The software manuals from Multi-Channel Systems (MCS; http://www.multichannelsystems.com/downloads/software) provide detailed instructions for software installation, setup and use. Here, we provide start-up instructions for the older MC_Rack software, but there is now the newer and improved MC_Suite program for data acquisition, visualization and basic analysis. If you choose to use MC_Rack, download MC_Data tool for data management. If you have also purchased a PPS2 and TC01/TC02 systems, installing the appropriate software (PPS2 and TCX-Control, respectively) will allow easy access and control of these devices via USB. Below includes an example of a useful way to build a "Rack", which is a recording configuration provided in the MC_Rack software (*see* Figs. 4-5).

4.1 Data acquisition: action potential/spikes recording

- Load the MC_Rack software
- In general, it is advisable to have the online display showing: 1) long-term and short-term (30s and 1s, respectively) raw unfiltered data streams; 2) high-pass filtered data (1s) with threshold-line indicator to isolate spikes (Figs. 4-5); and 3) multi-unit activity rate histograms (Fig. 4e). The latter are critical as they provide live second-by-second updates of your recording, alerting you of any recording problems. The online rate histograms also provide the perfect visual guide for appropriate timing of drug treatment, cues for duration of drug actions and recovery to baseline activity (Fig. 4e-*e1*).
- To create a Rack containing these displays, do the following: In the Rack, click on the hardware's name (e.g. MEA2100 (1/2) SN) and add Filter, Display, and Long-term display. Click on the Filter icon and add Spike sorter, Analyzer, and Parameter display (rate histograms). Click on Filter and choose the Butterworth 2nd order high-pass filter with cut-off frequency set at 300 Hz. One of the options in spike sorter allows you to set spike threshold (please refer to the appropriate part of the manual for more details). Once you have built a rack you can save it for future use. If you are running two pMEA chambers you will need to run two separate MC_Racks side by side. Ensure that each one communicates with the appropriate pMEA chamber.
- This system can sustain continuous high-speed USB data transfer with a maximum sampling rate of up to 50 kHz/channel (for most recordings we recommend using 25kHz). Although we strongly recommend saving the raw data files during

acquisition, this may not always be practical, especially for longer-term recordings (>10 hours). This is because raw data files acquiring at high sampling rate require large amount of storage space (>100 GB/day). One way to circumvent this is to record spikes and spike-time stamps only (at 50 kHz these require ~5GB/day). The down-side of this is you need to establish which online threshold values to use before you start recording. This can have disadvantages if you encounter a prolonged period of sporadic electrical noise during your recording, for example. In these circumstances you will not be able to re-threshold your spike data to exclude such noise after recording or in future analysis. Recording at lower sampling rate will also reduce storage space needed, but if too low the spike waveform will become distorted, thereby rendering spike sorting more difficult to achieve.

- At the end of your experiment apply tetrodotoxin (TTX, 1µM; Tocris) for at least 10 minutes. This silences AP discharge and produces an activity-free segment of the recording trace that can be used to guide analysis (See Figs. 4d, 4*d1*, 5d and 5*d1*, and **Note 14**).

4.2 Offline data extraction, assembly and analysis

- During your experiment, MC_Rack saves your data in discrete files. So, before extracting spike frequency and time-stamps you need to concatenate the files with MC_Data tool.
- Build a Rack as described above. Remember, this time you will be replaying and re-recording the data, extracting the spikes and spike time-stamps in the process (consult the manual) for further analysis. So, in your Rack, there must be an additional "replay" icon.
- Once extracted, spike frequency and time-stamps within the file can be read by other commercially available software for more sophisticate analysis, such as "NeuroExplorer" for multi-unit activity (MUA) frequency analysis and "Offline Sorter" for spike sorting (dissecting single-units from MUA; Plexon; http://www.plexon.com). The data can also be exported to MATLAB (https://uk.mathworks.com) and/or Python (https://www.python.org/)/ANACONDA https://www.continuum.io) for customized scripts-/algorithms-based analysis.
- MUA frequency analysis from large datasets is labor intensive and requires long periods of time to complete. Further, some of the commercially available software are

expensive and they are not designed for circadian data analysis in mind (such as accurate adjustment for Zeitgeber- or circadian-time, and/or cull-, and recording-time). We are preparing a MATLAB-based circadian tool for fast, user-friendly and systematic analysis of baseline MUA acquired across multiple brain slices over the circadian day. This tool also provides speedy and highly accurate measurements of acute drug effects on MUA across all 60 electrodes. This will be available in due course.

5. Drug tests

To investigate whether all aspects of the recording setup are operating as should be, we recommend performing a drug application test (e.g. Figs. 4e and *e1*). This allows the user to approximately time drug action-onset, duration, and washout (this depends on the drug being used). For most brain circuits, exogenous application of fast-acting/washout agonists such as (*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide (a water soluble form of AMPA) or γ-Aminobutyric acid (GABA) (Tocris) will do, as an initial one-off guide. We strongly recommend setting up the ramps of the stopcock Luer-lock system to avoid drug cross-contamination. This also provides accurate control when switching from control aCSF to drug-containing aCSF (See Figs. 1b and 2l). For our setup, and as a rule-of-thumb, most drugs should take 1-2 minutes to reach the pMEA chambers.

6. Shutdown and cleaning

- Capture and image your slices *in situ* (as shown in Figs. 4b and 5b).
- Stop the acquisition software.
- With a fine pair of forceps carefully remove the tissue anchor.
- Close Tap-C and -D (Position-A) to relieve the slices from the CVP suction. The gravity-led inflow should now start to lift the tissue off the pMEA chamber base, away from the electrode contacts.
- With a fine paintbrush lift the slices completely off the electrodes, removing them from the pMEA chamber. The slices can be used for further processing, such as immunocytochemistry.
- Close Tap-A and -B (Position-A or -B) and momentarily stop the PPS2 peristaltic pumps.

- Wash and switch the inflow tubes (upper and lower) to a 2L bottle filled with distilled water.
- Restart the upper perfusion by switching the PPS2 peristaltic pumps back on.
- Place Tap-A and -B in Position-A, and using the attached syringes withdraw fluid until the distilled water enters the syringes.
- Place Tap-A to -D in Position-C.
- Slowly the distilled water will replace the circulating aCSF. Run for at least one hour. We recommend running 70% ethanol through the tubing system for at least 10 minutes after each experiment to clear-off stubborn contaminants, especially if drug treatments were used.
- Withdraw the tubing ends from the distilled water and thoroughly drain the tubing.
- Open the MEA lid and carefully lift and remove the pMEA chambers.
- Remove the parafilm, if this has been applied, and store the pMEA chambers in 1% Tergazyme at 4^{o}C. Store the tissue anchor and blue rubber O-ring in distilled water at room temperature.
- Dry the perfusion elements, inspect the gold pins for signs of damage/spillage/aCSF salt residue, and close the MEA lid.
- Carefully and diligently inspect the area around the MEA head-stage, pumps, tubing etc., ensuring that they are clean and dry with no aCSF salt residue, and tubing/wires etc. are not out of place.

7. Notes and troubleshooting

1) Figs. 1-2 show how to setup two sealed reservoir-flow system. Use rubber bungs that can completely sealed-off the aCSF reservoirs, preventing dissolved gas escaping from the aCSF and leaking from the bottles (Figs. 1a and 2j). Bore three holes in each rubber bung, two for the tubing, and in the third insert a hollow glass tube (Figs. 1*a1* and 2j). This glass tube must be long enough to span the distance from the bung to near the base of the bottle. Connect this tube to a leak-resistant gas bag to equilibrate negative pressure build-up during aCSF flow (Figs. 1*a3* and 2i). Fill, but do not pressurize this bag with gas containing 95% O_2 and 5% CO_2.
2) Cut off the hub of four 18G hypodermic needles and file-down the sharp beveled end. Cut an appropriate section of the inflow tubes, ahead of the peristaltic pumps, and under inflows, and insert the needle shafts as shown in Fig. 1 (enlarged in 1*a4*).

Ensure that where you cut the tubes allows the tube-needle-tube assemblies to lie within the Faraday cage. Use this point to earth the system by appropriately attaching crocodile clips and earth wires (*see* **Note 3**).

3) These provide earth-points for aCSF leaving the MEA, reducing electrical noise. Attach these to crocodile clips and earth wires (*See* also **Notes 2 and 12**).
4) Any liquid overspills beyond the confinement of the blue rubber O-rings will introduce and magnify electrical noise, significantly reducing signal-to-noise-ratio. Excessive overspills can damage the electronic components of the MEA head-stage.
5) Using both hands gently press down on the MEA lid and slide-off the lid retainer/lock.
6) This is a critical step. Ensure that the blue rubber O-ring sits snuggly in the groove of the perfusion element. Failure to align this properly will cause the MEA to leak rendering it unusable (*see* **Note 4** and Figs. 1*d1*, 3b and 3c).
7) The pMEAs are stored in detergents. So, they must be properly washed before use. Place each pMEA in a small beaker filled with distilled water. Flush additional distilled water through the beakers for at least 2-3 minutes, emptying the beakers twice or three times during this process. Carefully examine the pMEA probes under a binocular microscope, ensuring that there are no residues or debris on the electrode terminals or contact pads. **DO NOT** at any point allow the pMEA electrodes to get totally dry.
8) The compactness of the MEA2100-HS2x60 system makes it extremely vulnerable to accidental flooding or stray water/aCSF spray/droplets, which if come into contact with any of the MEA's electronic components, including the gold pin contacts, will introduce noise. These floods/spray/droplets can cause long-term damage to the system. To circumvent this, we have developed an in-house method for adding an extra layer of protection against accidental spills. Excise a piece of parafilm (Bemis Company Ltd, USA: PM996) (~10x10 cm^2) and stretch it slightly. Center it over the pMEA chamber and fix in place by gripping a rubber O-ring around the outside wall of the chamber, firmly holding the parafilm in place (*see* sequence in Fig. 3g-i). For this to properly work, the internal diameter of the O-ring must be slightly less than the external diameter of the pMEA chamber. With a razor blade, cut-off the top section of the parafilm exposing the opening of the pMEA chamber (*See* Fig. 3g-i, and Figs. 3i and 3k show the finished outcome).

9) Place the aCSF reservoirs a few centimeters higher than the perfusion elements.
10) Besides using the magnetic perfusion holder, provide additional support/reinforcement to the cannula-PH01 temperature unit assemblies to ensure that these always remain in place. This can simply be done by applying “blu tack” (Bostik Ltd, UK) under the PH01 units.
11) Appropriate setting for this depends entirely on your setup and which pMEA chambers you are using (6x10 or 8x8). The optimum value can only be achieved *in situ* by trial and error during the initial one-off setup. Start with 42 mbar and increase this pressure if the tissue is not being firmly pulled-down onto the pMEA and hold in place. Success in achieving good contact between the slice and electrode can be identified visually from the level of AP activity emanating from the slices. Thicker sections need higher negative pressure. For longer recordings (more than 10 hours) err towards lower values. This is because the negative pressure does exert some mechanical hold on the tissue and lower suction values during long-term recordings ensure that the sections are not overly stretched or stressed. However, keep in mind that lower negative pressure settings may compromise the signal-to-noise ratio, but this slight offset in recording quality contributes towards slice longevity.
12) These values depend on how well the rig is earthed. How to properly and successfully earth a rig requires systematic checking. Electrical noise can be a real challenge to completely eliminate and requires experience. In Fig. 1 and **Note 2** we have indicated strategic locations along the setup that can be used as earth-points. These helps to maintain low electrical noise. Connect these earth-points to the Faraday cage and table top using crocodile clips and electrical wires. The best rule-of-thumb to avoid electrical noise is to always maintain the rig clean, dry and salt residue-free.
13) If your slice is in good physiological condition (alive and undamaged), but you cannot detect any AP discharge, this means that there is not enough contact between your slice and the pMEA electrodes. Slowly increase the CVP suction. If the issue is not resolved, you may have air bubbles trapped in the underflow tubing which are preventing negative pressure transfer from the CVP to the pMEA chamber and slice. Remove the slices from the pMEA chamber and use the syringes to try and rectify this. Please **NOTE** that the inflow and outflow/negative pressure for the under perfusion system need to be carefully balanced to accommodate for tissue thickness and recording duration (*see* **Note 11**).

14) TTX treatment at the end of your experiments confirms AP activity and provides a highly reliable and accurate way to determine signal threshold. Use this TTX segment of your recording to determine and set threshold level for spike extraction (*see* Figs. 4d, 4*d1*, 5d, and 5*d1*).

8. Conclusion

Here, we provide a step-by-step procedural overview for setting up and recording from brain slices using pMEAs. In particular, we describe fitting methods for the new MEA2100-HS2x60 system, but similar approaches can be used to setup and record with other pMEA devices currently available. We find that this way of the pMEA system assembly, regimented setup and recording methods provide stable, highly-reproducible and high-quality recordings that can last for several hours. When appropriately fine-tuned (*see* **Note** 11), this setup method can be used to interrogate neuronal activity well over the circadian day.

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11. Figure legends

Fig. 1. Schematic layout for our pMEA system setup. **a**) aCSF reservoirs, **b**) drug delivery reservoirs, **c**) PPS2 peristaltic pumps, **d**) pMEA head-stage unit with dual-recording capability (labelled as 1 and 2). Inset (***d1***) shows the cross-section of a pMEA-perfusion element assembly *in situ*. **e**) CVP pump, **f**) MEA2100-interface board, **g**) 2-channel temperature controlling unit (TC02), and **h**) acquisition computer. Perfusion tubes are colour coded to indicate upper and lower inflow and outflow systems. Blue arrows indicate the direction of perfusion flow (*see* also Figs. 3j and 3k). ***a1***) shows the hollow glass tubes, ***a2*)** the inflow tubing, and ***a3*)** the tubes that supply 95% O_2 and 5% CO_2 contained in the gas bag to the bottles via the hollow glass tubes. This assembly offsets the vacuum that builds inside the bottles as the aCSF drains. A Tap configuration key is provided with red arrows indicating directions of solution flow when syringe plunger is pulled or pushed for suction and flushing, respectively. ***i)*** shows a diagram of the syringe attached to a 3-way tap, and ***ii)*** Tap Position-A to -C configurations as referred to in the text. Thick green arrows indicate points where earth wires fitted with crocodile clips can be attached, with inset ***a4***) showing an

enlarged diagram of such tubing-earth points. PPS2: 2 peristaltic perfusion system; PH01: heatable perfusion cannulas with temperature sensor; TC02: 2-channel temperature controller; CVP: Constant Vacuum Pump; MPH: magnetic perfusion holder. *See* Fig. 2 for a photograph of the setup with associated labelling.

Fig. 2. Photograph of our pMEA system setup. Letters (**a** to **h**) identify the components shown schematically in Fig. 1. **i**), gas bag, **j**) rubber bung with hollow glass tube and tubing assembly, **k**) water bath, **l**) ramps of stopcocks Luer-lock for drug delivery, **m**) heatable cannulas (PH01), **n**) syringe-Tap assemblies for under perfusion system (*see* Fig. 1 for Tap-A to -D setup), **o**) gooseneck of light source, and **p**) binocular microscope.

Fig. 3. Photograph of the pMEA and parafilm protection lay-down process, and over perfusion system setup. **a**) shows one of the two perfusion elements with ***a1*** identifying the grove/notch, ***a2*** and ***a3*** showing inlet and outlet of the under perfusion system, respectively, **b**) positioning of the blue rubber O-ring (***b1***) in the grove/notch, **c**) shows the O-ring snuggly in place in the grove with (***c1***) demonstrating the water pool referred to as 3c in the text. This water pool becomes continuous within the inlet and outlet tubing (from the reservoir bottle to the CVP pump: see text), **d**) depicts the cover slipping action during the pMEA lay-down process, showing the leading water edge (***d1***), **e**) displays the pMEA properly laid-down onto the blue rubber O-ring with no air bubbles and overspills, and **f**) showing an example of a poorly laid array, indicating a large pocket of air (***f1***) trapped underneath the pMEA chamber. ***e1***) shows the electrode contact pads (where the electrodes form connection with the head-stage). **g** to **i**) show the parafilm setting up process with **j** and **k**) showing the complete over perfusion setup without (**j**) and with (**k**) parafilm protection in place. Blue arrows indicate direction of over perfusion inflow and outflow. ***j1***) are the magnetic perfusion holders (MPH), and ***k1-2***) showing the tissue anchor (***k1***) and slice (***k2***) assembly. ***k3)*** shows the beveled end of the suction cannula. *See* also Fig. 1. REF: reference electrode.

Fig. 4. Photograph of 6x10 (**a**) pMEA electrode layout. Notice the perforation lattices of various sizes distributed among the electrodes. **b**) Photograph of SCN slice being recorded *in situ* on a 6x10 pMEA probe. Notice that the pMEA electrodes can be visually identified from underneath the slice. **c**) 1s snapshot of extracellular action potential (AP) discharge at the electrodes from the SCN slice shown in (**b**). APs are highlighted as they cross the threshold line (here set at -17.5 µV, shown as enlarged inset in (***c1***)**. d**) shows 1s snapshot of the

recording during 1 µM TTX application (~5-10 min), indicating the absence of APs. Notice the threshold lines in black underneath the traces. These can be individually set or the same value can be applied to all channels. **e**) shows time series rate histograms (Hz) obtained from measuring AP frequency over 800s with the acute rise in activity depicting examples of AMPA treatment which transiently excited neurons across the SCN slice. One channel of this located in the ventral SCN (marked with a green box) is enlarged in ***e1*** with top black line indicating the duration of the AMPA application (150s). ***c1*** and ***d1***) are enlarged insets of channels taken at regions marked by the blue boxes in **c** and **d**, respectively, showing a 1s recording during control aCSF (***c1***) and in aCSF containing TTX (***d1***). ***c2*** shows an example of SCN multi-unit AP activity waveforms. REF: reference electrode; *t (*in ***d1)***: example of threshold line.

Fig. 5. Photograph of 8x8 (**a**) pMEA electrode layout. Notice the perforation lattices of various sizes distributed among the electrodes. **b**) Photograph of MBH slice being recorded *in situ* on a 8x8 pMEA probe. Notice that the pMEA electrodes can be visually identified from underneath the slices. **c**) 1s snapshot of extracellular action potential (AP) discharge at the electrodes from the MBH slice shown in (**b**). APs are highlighted as they cross the threshold line (here set at -15.5 µV, shown as enlarged inset in ***c1*****). d**) shows 1s snapshot of the recordings during 1 µM TTX application (~5-10 min), indicating the absence of APs. ***c1*** and ***d1***) are enlarged insets of channels taken at regions marked by the blue boxes in **c** and **d**, respectively, showing a 1s recording during control aCSF (***c1***) and in aCSF containing TTX (***d1***). Notice the threshold lines in black underneath the traces. These can be individually set or the same value can be applied to all channels. ***c2*** shows an example of MBH multi-unit AP activity waveforms. REF: reference electrode; *t (*in ***d1)***: example of threshold line.