SAND MEA Plating & Cell Culture Protocol

Brief summary: This protocol includes the steps for preparing, plating, and culturing neurogenin-2 (NGN2) induced pluripotent stem cell (iPSC)-derived human cortical neurons on single-well microelectrode arrays (MEAs) from Multichannel Systems and multi-well plates from Axion Biosystems. This protocol assumes a stock of frozen days-in-vitro (DIV) 4 neurons. However, this protocol can easily be accommodated to plate non-frozen cells harvested at DIV 4-10 after differentiation from iPSCs to neurons.

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I. MEA Preparation

A. Cleaning MEAs for Reuse

Sterile MEAs in the Incubator

1. If MEAs are sterile in the incubator, the first step is to remove the cellular debris from previous cell culture on the MEA using Proteinase K. In the biosafety cabinet, aspirate most of the media in the well and add ~200 µl of sterile (filtered) proteinase K (enough to cover the entire MEA well) to each MEA. The proteinase K is activated by calcium; thus, having a thin layer of media left—not enough to dilute proteinase K—is helpful. *Warning: Proteinase K digests hair, skin, and nails. Be careful not to get any proteinase K in other areas of the biosafety cabinet. If you get on your gloves, put them in the autoclavable waste before touching anything else. Any paper towels that you use to clean up the proteinase K should also go in the autoclave bag. Do not put it in regular trash! Proteinase K is inactivated by high heat.*

2. Replace MEA lids (also called covers) and return the MEAs to the incubator for 24 hours.

3. After 24 hours, check MEAs under a microscope to see if the debris is gone. If not, return them to the incubator for another 12-24 hours. You can also use manual pipetting to help dislodge debris; however, care must be taken not to touch the bottom of the MEA well with the pipette tip as this can permanently damage the MEA.

4. To remove proteinase K from MEAs, use a 1 mL pipette and place it in a labeled 15 mL centrifuge tube. The 15 mL tube will be disposed of in the autoclavable waste.

5. Add ~500 µl of sterile water to each MEA and then use the 1mL pipette to remove contents and place in the labeled 15 mL tube. Close the tube tightly once done. Dispose of the tube in the autoclavable waste, or a hazardous waste bucket, depending on your institution's policies.

Non Sterile MEAs

1. If MEAs have been removed from the incubator, the first step is to remove any large debris.

2. Clean used MEAs with sterile deionized water at the sink to remove any debris.

3. Carefully add ~200 µl Proteinase K to the MEAs. MEAs should be placed inside their Petri dishes to avoid contaminating countertops with proteinase K. With a sign to warn other lab members of proteinase K, leave the wells for 24 hours. *Warning: Proteinase K digests hair, skin, and nails. Be careful not to get any proteinase K in other areas of the counter top. If you get on your gloves, put them in the autoclavable waste before touching anything else. Any paper towels that you use to clean up the proteinase K should also go in the autoclave bag. Do not put it in regular trash! Proteinase K is inactivated by high heat.*

4. To remove proteinase K from MEAs, use a plastic Pasteur pipette to remove Proteinase K and place it in a labeled 25 mL tube. This 25 mL tube will be disposed of in the autoclavable, or hazardous, waste depending on your institution's policies.

5. Fill each MEA well ~30-50% full of distilled water and then use the plastic Pasteur pipette to remove contents and place in the labeled 25 mL tube. Close the tube tightly once done. Dispose of the tube in the hazardous waste bucket. Make sure the hazard waste container's lid is sealed when done.

B. Sterilizing MEAs

1. For non-sterile MEAs after cleaning, soak single-well MEAs in sterile, deionized water in a plastic box (we use sterile tip box after all the tips are used) to fully clean for 15 minutes up to an hour. Multiwell plates can be filled with water.

2. Let MEAs dry on a lint free paper towel on the counter. Dispose of water in the sink.

3. Once the single-well Multichannel Systems (MCS) MEAs are fully dry they can be sealed in autoclave bags. **Multi-well plates (or single-well MEAs that cannot be autoclaved) should be soaked in 70% ethanol and transferred to the biosafety cabinet to dry.** If the single-well MEAs will not be reused within

a few days, they can be stored in petri dishes on the counter and labeled “ready for autoclave.”

4. One to two days before a culture initiation, seal the single-well MEAs in an autoclave bag (and lids separately). Sterilize in the autoclave on the liquid cycle. Follow instructions for using the autoclave. For our autoclave, see details in another protocol titled “MEA Autoclave.”

N.B. If there is a concern for fungal infection, the MEAs and lids can be treated with hydrogen peroxide prior to autoclaving. For the MEAs, use an empty tip box and briefly submerged the MEA in 30% hydrogen peroxide (wear full personal protective equipment). Add distilled, deionized water (ddH_2O) to dilute the hydrogen peroxide to 3% (percentage that is used to sterile lacerations clinically) and then you can use a gloved hand to remove MEAs and put in an empty (clean) tip box with ddH_2O to further dilute (remove) the hydrogen peroxide. Once clean the MEAs should be autoclaved. The membrane and rubber seals need to be removed first and disposed of for the lids (covers). Then the white lids can be soaked in the hydrogen peroxide for 10-30 minutes. The membrane and seals need to be replaced before autoclaving. Do not mix the MEAs that might have fungus with any MEAs that are fungal-free. Fungal spores can easily transfer on contact from one surface to another and are difficult to get rid of (hydrogen peroxide is good but not 100% effective against spores). Alternatively, you can heat shock spores by autoclaving first and then soaking in hydrogen peroxide.

C. MEA Pretreatment

Transferring Autoclaved Single-well MEAs to Biosafety Cabinet

1. We use sterile petri dishes to keep the single-well MEAs and incubator clean and to prevent damage to the MEAs. Open the sterile petri dish package in the biosafety cabinet and remove the necessary numbers of dishes. Tape the bag with the remaining petri dishes securely to keep them sterile and remove the bag from the biosafety cabinet. Label the petri dish bag “STERILE + your initials + date.”

2. Use 70% ethanol to spray and wipe the autoclave bags with MEAs and lids prior to placing the sealed autoclave bags upside down in the biosafety cabinet to dry (paper side up). Open the autoclave bag in the biosafety cabinet while holding it with one hand and using another hand to pull back the cover. *N.B. If*

using nylon autoclave bags with tape, cut open the bag with small scissors sterilized with 70% ethanol. Take care not to cut the autoclave tape as this may transfer adhesive to the scissors/biosafety cabinet surface.

3. Change gloves and spray gloves well with 70% ethanol well and let gloved hands dry in the biosafety cabinet before touching MEAs or lids. Transfer MEAs to the Petri dishes gently, without touching the ring or wells.

4. Next remove MEA lids from autoclave bags carefully and fit each MEA with a lid. Extra lids can be stored in the sterile box in the biosafety cabinet.

5. Label each petri dish with the MEA serial number (on the bottom of the MEA), the date of the experiment, and your initials.

Making the MEA Hydrophilic with PLL (or PEI)

Background: New MEAs are typically hydrophobic and may also become hydrophobic during storage. This may prevent the attachment of the hydrophilic cells. Thus, the first step in pre-treating the MEAs is to ensure that the surface is hydrophilic enough for coating and cell adhesion. To achieve this goal, we coat the MEA grid only with high molecular weight poly-L-lysine(PLL). PLL is preferred over polyethyleneimine (PEI) as it can be purchased inexpensively, already sterile. MEAs with PLL can also be kept in the incubator in contrast to PEI which needs to be kept in the fridge overnight. Instructions for PEI are provided after PLL. Do not use both treatments.

Poly-L-lysine (PLL) Treatment

1. You will need 5µl of PLL for each MCS single-well 60-electrode MEA or for each well of the Axion 48-well 16-electrode MEA plates. You will need 7µl per well for the Axion 6-well MEA plate due to the larger MEA grid size. Using a 20µl pipette, hold the pipette near horizontal (parallel to the surface of the biosafety cabinet surface) and lower the pipette tip to ~5mm directly above the grid (center of the MEA). *Do not touch the surface of the MEA well with the tip!* Slowly expel the PLL to form a drop at the tip of the pipette. Carefully lower the tip until the drop of PLL at the end of the tip touches the MEA grid and expands, pulling the pipette tip quickly away to avoid touching the grid with the tip. A new pipette tip should be used for each MEA to avoid cross-contamination.

2. Incubate the PLL-coated MEAs in a cell culture incubator at 37°C, 5% CO2 for 5 minutes up to 24 hours. The time necessary will depend on how hydrophobic the surface is. If reusing MEAs, 30 minutes is typically all that is necessary. For new MEAs, 24 hours is best. If you place a drop of sterile water on the MEA surface and it runs away, the surface is very hydrophobic. If the water spreads out, it is very hydrophilic.

3. After incubating in PLL, remove PLL by washing three times with 20 µl of sterile PBS (no Ca, no Mg) or sterile water 3 times with the same method as described in Step 1 of the PLL treatment. *We use PBS if we will add the laminin within 30 minutes of completing this step. We use sterile water if there is expected to be a delay of 30 minutes up to 24 hours before laminin will be added.*Allow at least 5 minutes to pass before removing the first wash if the PLL treatment was applied for more than an hour. Remove the PBS or water each time using the suction with a sterile (no filter) 200µl tip to aspirate. Then allow the MEA plates to dry in the incubator. *Warning: Failure to remove all of the PLL can be toxic to the cells.*

Polyethyleneimine (PEI) Treatment (alternative to PLL)

1. Prepare a stock solution of 0.1 % PEI dissolved in distilled water or borate buffer. The borate buffer includes: 1.24 g boric acid, 1.9 g borax (sodium tetraborate) and 400 ml distilled water.

2. You will need 5µl of PEI for each MCS single-well 60-electrode MEA or for each well of the Axion 48-well 16-electrode MEA plates. You will need 7µl per well for the Axion 6-well MEA plate due to the larger MEA grid size. Using a 20µl pipette, hold the pipette near horizontal (parallel to the surface of the biosafety cabinet surface) and lower the pipette tip to ~5mm directly above the grid (center of the MEA). *Warning: Do not touch the surface of the MEA well with the tip!* Slowly expel the PEI to form a drop at the tip of the pipette. Carefully lower the tip until the drop of PEI at the end of the tip touches the MEA grid and expands, pulling the pipette tip quickly away to avoid touching the grid with the tip. A new pipette tip should be used for each MEA to avoid cross-contamination.

3. In the biosafety cabinet, cover MEA petri dishes (single-well) or plates (multi-well) completely with parafilm that has been cleaned with 70% ethanol—to keep the MEA plates or petri dishes sterile. Place the

parafilm-wrapped MEAs plates/dishes in the 4 °C fridge overnight. The next day carefully wipe the parafilm with 70% ethanol. Remove parafilm in the biosafety cabinet, taking care to keep the MEA plates/dishes sterile. *N.B. This is the main reason that heavyweight PLL is preferred over PEI. The PLL is sterile and the plates and dishes can be in the incubator overnight (cleaner than fridge).*

4. Remove PEI from the MEA by washing with 20 µl of sterile water 3 times with the same method as described in the Step 2 of the PEI treatment. *We use PBS if we will add the laminin within 30 minutes of completing this step. We use sterile water if there is expected to be a delay of 30 minutes up to 24 hours before laminin will be added.* Allow at least 5 minutes to pass before removing the first wash if the PLL treatment was applied for more than an hour. Remove the PBS or water each time using the suction with a sterile (no filter) 200µl tip to aspirate. Then allow the MEA plates to dry in the incubator. *Warning: Failure to remove all of the PEI (pH ~9.5) can be toxic to the cells.*

Laminin for Long-term Cell Adhesion

Background: Laminin is an extracellular matrix glycoprotein that is applied as a thin coating layer on the MEA to promote cell adhesion. It comes in a frozen vial and should be stored in a -20 °C freezer in small aliquots.

1. Laminin should be added to the MEA grid within 30 minutes of when the cells will be plated. This improves cell adhesion to the MEA grid.

2. Thaw the laminin aliquot immediately before use. Avoid extended exposure of the protein to ambient temperatures. There is a short interval between the laminin thawing and becoming too viscous to pipette. When there are many MEAs to treat with laminin, the laminin stock solution should be kept on ice (or aliquots can be removed from the freezer as needed).

3. For MCS single-well 60-electrode MEAs, we apply 12µl of laminin per MEA as drop over the center of the MEA (needs to completely and evenly cover the grid). We use the same technique for adding the laminin to the MEA grid as used for the PLL (or PEI) and PBS (or water) treatments. For larger MEA grid sizes such as custom single-well 60-electrode MEAs, we use 15-20µl per MEA depending on the grid size (confirm complete coverage of the grid under microscope). For the Axion 6-well MEA plates, we use a 20µl laminin per well. pipette and remove the desired concentration. For the smaller 48-well MEA plates with 16-electrode MEAs, 7-10µl laminin per well is used (confirm coverage

of MEA grid under microscope). *Warning: Do not touch the pipette tip to the MEA surface!*

4. After laminin is added, attach the MEA lid and return MEAs to the incubator. Plate the cells within 30 minutes of laminin coating to avoid the laminin drying.

II. Neuronal Cell Preparation

1. We use days-in-vitro (DIV) 4 frozen human iPSC-derived neurogenin-2 (NGN2) cortical neurons provided by the BWH iPSC NeuroHub. When using fresh cells (not frozen), skip to the cell counting step (8) below.
N.B. For investigators at BWH, cells can be requested at (https://neurohub.bwh.harvard.edu/cell-requests.html). Include the cell line (e.g., BR33) and the number of vials you need. Requests should be made ideally a week in advance. You will receive an email with details on pick-up date and location, once the cells are ready. The vial should be transported on dry ice to the -80 °C freezer if using them within a few days. Otherwise, it should be kept in a -150 °C freezer (see M.K. for assistance).

2. Warm the re-suspension media, complete neurobasal media (NBM) with 1:1000 ROCK inhibitor (ROCKi), for 20-30 minutes to 37 °C. This can be done using the heater in the biosafety cabinet or the water bath. If the latter, wipe well with 70% ethanol before transferring to the biosafety cabinet.

3. Label a 15-mL centrifuge tube with cell/line number “BR33 Cells” and add 3 ml of the warmed media. If multiple cell lines, label one tube per cell line.

4. Thaw the cells quickly by gently swirling the lower half of the vial in a 37 °C water bath. Do not submerge the vial completely. Observe the vial closely during the thawing process. Once a small amount of ice is left in the vial (typically takes less than 2 minutes), remove it from the warm bath. Quickly dry and thoroughly clean with 70% alcohol before transferring the vial to the biosafety cabinet.

5. Remove the vial cap. Without touching the rim, use a 1 ml pipette to suspend cells with 1 ml of the warm media. Add the warm media to the cells slowly in a dropwise fashion. *Warning: Adding the media dropwise and pipetting style is key here to cell survival. Too rapid shifts or too intense*

pipetting can kill cells. Avoid creating bubbles while pipetting and transferring the cells.

6. Dropwise, transfer the cells from the vial to the 15-mL centrifuge tube you labeled with cell line (step 3). Use 1mL more of the warmed media to rinse the vial, and pipette up and down a few times to make sure all the cells are removed from the vial. Add that 1 mL to the 15-mL tube with the cells. The 15-mL tube with the cells will now have 5 ml of media total (along with the cells). *Warning: Avoid creating bubbles while pipetting and transferring the cells.*

7. Label one 600µL sterile PCR tube for each cell line. Add 10µL of cell suspension with 10µL Trypan Blue Stain (0.4%).

8. Centrifuge the cells in the 15-mL tube at 200 × g (rcf) for 5 minutes.

9. While the cells are in the centrifuge, transfer 10µL of the mix of cells and trypan blue to the cell counter slide. Follow the instruction of the cell counter to count the cells. Write down cell counts and percent viability on a piece of paper that can be transferred to the lab book. *N.B. If the viability is less than 80%, consider thawing a second vial to get a higher viability. This is important as a large number of dead cells on the MEA affects the recordings.*

10. Calculate the volume of media to resuspend the cells such that 15µL of the resuspended cells in media will have the desired concentration of cells. For the laminin drop sizes recommended in this protocol, we recommend plating 35,000 cells per MEA. *Warning: This step is time sensitive. You need to finish before the centrifuge is done!*

The calculation is based on the number of live cells (as counted by the Countess 3 Cell Counter). Write down each step and double check calculations.

$$\frac{\text{Total number of cells (in 15mL tube)}}{\text{? volume to resuspend}} = \frac{\text{Desired number of cells per MEA}}{\text{15µL drop to plate on MEA}}$$

Thus, you can multiply the number of the viable cells times 15µL and then divide by the desired concentration (e.g., 35000). As long as you keep track of

your units, you should get the volume to resuspend in µL if you use 15µL (or in mL if you used 0.015 mL) in your calculation. Write this volume down for each line.

11. Confirm there is a pellet at the bottom of the tube when removing the tube from the centrifuge (if not spin again). Gently clean the tube with 70% ethanol before returning the tube to the biosafety cabinet.

12. In the biosafety cabinet, use a 1 mL pipette to gently aspirate the supernatant. Immediate resuspend the cell pellet in the volume of warm media calculated in step 10. Once resuspended, proceed immediately to plating the cells on the MEAs. *Warning: Any delay in this step can decrease cell viability. Carefully pipetting up and down to fully resuspend is important to avoid clumps of cells but also carefully enough to reduce shearing effects is critical for cell survival. This is the step that requires practice (and ideally feedback from scientists skilled in working with iPSCs.*

III. Plating Neuronal cells on MEAs

1. Gently mix the tube of cells with the 1-mL pipette thoroughly to ensure cells are not clumped.

2. Use the 20-µL pipette to take the required volume of cells (e.g., 15µL per well) and hold the pipette near horizontal with the pipette tip directly over the laminin drop at the center of the MEA. Slowly pipette the cells into the laminin drop for each MEA. A new pipette tip should be used for each MEA to avoid cross-contamination. *Warning: Do not touch the pipette tip to the MEA surface! This step is time sensitive. If you take too long to plate the cells, their viability will decrease. If you pipette too quickly or too frequently, the shearing force will lead to cell death. If you do not sufficiently mix the cells, the cells will clump on the MEA affecting the culture development.*

3. After cells are plated on the MEAs (or MEA plate), the MEAs should be immediately returned to the incubator. A timer should be set for 20 minutes. The cells need at least 20 minutes (but not more than 30 minutes or they will dry out) to adhere to the MEA before the remaining volume of media. *N.B. If multiple multi-well MEA plates are being plated, we recommend setting one timer per plate to keep track of the precise timing.*

4. Save any extra cells in the fridge at 4 °C until cell adhesion is confirmed in Step V. After this step, any extra cells can be discarded in the biomedical waste.

IV. Plating Astrocyte on MEAs

If co-culturing with human astrocytes, prepare the astrocytes in advance (see Astrocyte Culturing and Preparation protocol). Due to the time critical nature between neuronal cell plating and adding the remaining volume of media to the MEA wells, the addition of astrocytes to the MEA must be completed within 20 minutes or else it threatens the neuronal cell viability on the MEA.

1. The astrocytes should be at least 70% confluent and passaged immediately before the laminin is applied to the MEA (Step IC). Remove 10µL prior to centrifuging cells for cell counting (as described in Step II).

2. While cells are in the centrifuge, use the cell counter to count the astrocytes using the same method as described in Step II. Calculate the resuspension volume such that 20µL of cells in neuronal media would contain 12,000 astrocytes.

3. Temporarily store the astrocytes in a centrifuge tube at 4 °C in the fridge.

4. Remove astrocytes from the fridge after neurons are plated (Step III). Use a 20-µL pipette and the MEA plating technique described in Steps IC and III to add 3-4 drops (~5-6.7µl each) evenly around—but not touching—the drop of laminin with the neurons on the MEA grid. These astrocytes “satellites” will help support the neurons without changing the number of neurons on the MEA grid.

N.B. Alternatively, astrocytes could be plated the day after the neuronal cell plating. In this case, after removing half of the media (contains ROCKi), add 20µL of astrocyte to each well directly into the media over the center of the MEA. Return MEAs to the incubator for 30 minutes to let astrocytes settle. Gently add back half of the media (no ROCKi).

V. Confirming Cell Adhesion and Adding Media

1. While the MEAs with the neurons plated are in the incubator for 20 minutes, keep the plating media (CNBM + 1:1000 ROCKi) at 37 °C in the water bath (or heater).

2. Check the MEAs under the microscope to confirm the neuronal cells are covering the MEA grid in similar numbers. Note that any floating cells may settle later (on or off of the MEA grid). *N.B. If there are insufficient cells, more cells can be added to the MEA grid. However, this does not always result in successful cultures. If cells are added, record which MEAs (wells) cells were added and how many cells were added. The number of living cells on the MEA grid impacts the maturation of the neuronal network development.*

3. Once the neuronal coverage of the MEA grid is confirmed, add the desired amount of plating media to each well. Carefully add in drops around the sides until the fluid coverages to avoid moving the cells off of the MEA grid. The MCS single-well MEAs require 600µL. We typically start with 750µL per well for the Axion 6-well MEA plates. We use 300µL for the Axion 48-well MEA plates.

4. Place the lid gently on the MEAs (and the lids on the Petri dishes for the single-well MEAs) and gently stack the plates in the incubator. *Warning: Avoid sudden or back and forth movements when transferring the plates. You do not want the cells to move off the MEA grid.*

VI. Maintenance of Long-term Cultures on MEAs

1. On the next day after plating (DIV 1) remove half of the media and replace it with the same volume of 37 °C feeding media to start the removal of the ROCKi. The feeding media is CNBM and should not contain ROCKi. *Warning: Remove and add media from the side of the MEA near the well wall. Remove and add media gently to avoid creating fluid movements that could remove cells adhering to the MEA grid. Removing nearly all of the media (as often done in non-MEA experiments) to get rid of the ROCKi has the high risk of dislodging the cells from the MEA grid. Thus, we only recommend removing half DIV1.*

2. Calculate the volume of the media required for feeding the cells for the week. *Warning: Do not use media if its color has changed. The media has a phenol red, a pH indicator that changes color with the change in pH. Neurons do not like big shifts in pH!*

3. We typically exchange the media three times per week by first aspirating the ~30-33% of media with a 200-µL pipette and then replacing the same amount with fresh 37 °C media. If there has been evaporation, more media can be added to keep desired total volume (this can happen on the single-well MEAs if the lid is not well sealed). *Warning: Avoid removing too much media that could expose the cells to the air and decrease viability. When adding media, do not pipette near or directly over the MEA grid (center of well).* As the cells mature, monitor the media color between media exchanges and increase the frequency, if needed, to keep the cells in a consistent healthy pH range. *N.B. Cells that are metabolizing nutrients lead to the media becoming more of a harvest gold color due to the acidity of the cellular metabolic waste products. If cultures stay pink or are more fuschia in color, this indicates more basic pH and is typically seen when cultures are very slowly metabolizing or when there has been significant (or complete) cellular loss.*

4. For the Axion 6-well plates, we will gradually increase the total volume of media in the well from 750µL to 1.5mL if we are keeping the cultures longer than 3-4 weeks. *N.B. Due to the large well size, compared to the MEA size, larger volumes of media at the beginning of the culture can dilute the growth factors produced by the cells and delay maturation.* Watch the media color between exchanges, increase the volume of media with subsequent media exchanges to keep the cells in a consistent healthy pH range.

VII. MEA Recordings

1. *When to start recording:* For human iPSC-derived NGN-2 cortical neuronal cultures, we typically do not see action potentials until the 2nd week in culture. Thus, we typically start recording at DIV 14. Short recordings of 1 minute in duration at DIV7 can be informative to see if the background noise level is similar in all electrodes and across different wells. If some wells show much higher standard deviation, it is important to look under the microscope and confirm coverage of the MEAs with cells and if the cells appear healthy or dead. Media (no cells) and dead cells have different impedance than living cells on the electrodes and will generally lead to higher variation in the voltage signal.

2. *When to ground electrodes*: Electrodes or wells with no cells or dead cells on all or a significant portion of the MEA should be excluded from downstream analysis. However, in the Axion MEA systems, the electrodes do not need to be grounded prior to recording unless they are leading to noise in neighboring electrodes (cross-talk). Grounding electrodes before recording in the Axion MEA Systems can lead to problems with using MEA-NAP, our MEA network

analysis pipeline. Instead, we recommend indicating which electrodes should be grounded in the batch analysis .csv file when using MEA-NAP. When exporting the Axion .raw files to .mat for the pipeline, it is important to remove any wells in which there were no living cells on the MEA. For Multichannel Systems (MCS) MEA recordings, electrodes with variation in the voltage signal greater than +/-50µV. If multiple electrodes are noisy, take the MEA chip or plate out of the MEA system and gently wipe the contact points around the side of the chip for MCS, and on the underside of the plate for Axion, with a lint free wipe lightly sprayed with 70% ethanol. Excess ethanol can block contact between pins (in the MEA system) and contact points on the MEA chip or plate. Media residue, for example transferred from gloves during media exchange) can also create salt bridges on contact points. Cleaning contact points can improve signal.

3. *Data acquisition settings:* We acquire data from the MCS MEA system at 25,000 Hz. For the Axion Biosystems MEA System, we acquire data at 12,500 Hz (maximum acquisition rate). These higher acquisition rates are necessary for using the template-based spike detection, which has higher sensitivity and specificity for many MEA culture preparations. The data will be filtered in MEA-NAP; thus, it is not necessary to add additional filters (beyond system standards) when acquiring data. We use a temperature recording to maintain the MEAs at 37 °C.

4. *Length of recordings:* We typically record for 10 minutes to sample the network activity. Shorter recordings may be sufficient for some experimental questions. However, if longer than 10-minute recordings are desired. We recommend recording in sequential 10-minute recordings so that the file size is still manageable for conversion and analysis on a desktop computer. If recording for longer than 10-12 minutes, we recommend using a perfusion system with 5% CO_2 or a HEPES-based ACSF to maintain pH balance.

5. *Naming recordings*: Systematic naming of files is essential for data management. With MCS Multichannel Experimenter acquisition software, the recording name needs to be confirmed prior to recording as the software saves multiple files which internally reference the original name. We recommend using a 3 or 4 alphanumeric prefix for the entire experiment (e.g., NGN2) followed by the date of the culture initiation to keep track of different batches (e.g., 241101, year-month-date will sort files by date later easily), then a specific identifier for the culture (e.g., “P1” for plate 1 for multiwell, or “A” for single wells that were assigned letters), and then the day-in-vitro with 2-digits (unless plan to go past DIV99, then could use three digits). N.B. When the Axion .raw

recording file (contains all wells) is unpacked by MEA-NAP during the file conversions, the well identification for each MEA will be added to the end (e.g., A1). Following this convention accurately labels the data and MEA-NAP outputs.

Sample filename: NGN2_241101_P1_DIV14_A1

6. *Ensuring sufficient space on hard drive:* Check to see there is enough hard drive space for the recording before you start (e.g., Axion 48-well plate 10-minute recording can be 11 GB). Otherwise the system will stop in the middle of your recording when it runs out of space.

7. *When to start recording (after data acquisition begins):* Make sure that sufficient acquisition time has passed for the voltage signal mean to be accurately calculated in all electrodes before you start recording. The Axion system has a gray bar moving along the bottom to indicate this process (typically takes less than 20 seconds). The MCS MEA 2100 system also needs about 10-20 seconds typically and this can be assessed by visual inspection of the voltage traces. With some custom MEA chips on the MCS, in contrast, it can take more than 10-minutes. For the older MCS MEA 1600 system, this process typically took the amplifier 2 minutes before a recording could start.

8. *Understanding limits of online spike detection*: Most MEA acquisition software, including those included with the MCS or Axion MEA systems, provide an estimate of spiking activity (action potential firing) during the MEA recording. This is typically achieved with threshold-based spike detection based on a standard deviation, absolute voltage, or in some cases can be manually set. It is important to understand that action potentials are only one of the sources for the variation in the voltage signal. Electrical and mechanical noise can also contribute. There are also the technical limitations of the electrodes to precisely detect the voltage. It is important to understand that if a standard deviation is selected for spike detection, that even when there are no action potentials, a specific percentage of the time “spikes” (i.e., voltage deflections that exceed the threshold). It is important to look at the waveforms and other information to accurately gauge if there is activity. MEA-NAP has a number of tools to aid in assessment of accurate spike detection.

9. *Sources of electrical and mechanical noise:* Do not move near the MEA system headstage or move the cables during recording. Do not jump, shut the door to the room, or make other movements that can create mechanical or electrical noise. If any of these events occur, record in your log book. Also note if noisy (e.g., nearby construction or someone running electrical equipment on

the same circuit) and consider waiting to record until a later time when noise is less. Anti-vibration tables and Faraday cages can reduce these types of noise. However, they are not available for all setups.

10. *How to stop recordings*: Use the automated settings such that you can start a recording manually but it will stop automatically at the end of the desired length of recording. This will give uniform file sizes, which is convenient for many types of downstream analysis.

11. *Converting files for downstream analysis in MEA-NAP:* The single-well recordings on the MCS MEA systems acquired with MC_Rack software require conversion first to .raw files using MC_DataTool and then to .mat using File Conversion in MEA-NAP. For acquisition with Multichannel Experimenter software, .mrsd files must first be converted to .h5 with Multichannel Data Manager and then to .mat using File Conversion in MEA-NAP. Axion MEA systems produce .raw files (not the same as MC_DataTool output also called .raw), which can be converted to .mat files using File Conversion in MEA-NAP.

If you use this protocol in your research, please cite our paper in *Cell Reports Methods*.

Sit, Feord, et al. (2024) MEA-NAP: A flexible network analysis pipeline for neuronal 2D and 3D organoid multielectrode recordings. *Cell Reports Methods*, 4(11), 100901.