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Establishment of an Electrophysiological Platform for Modeling ALS with Regionally-Specific Human Pluripotent Stem Cell-Derived Astrocytes and Neurons

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TITLE:

Establishment of an Electrophysiological Platform for Modeling ALS with Regionally-Specific Human Pluripotent Stem Cell-Derived Astrocytes and Neurons

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KEYWORDS:

multi-electrode array; extracellular recording; screening; assay; spinal cord; glia; motor neuron; amyotrophic lateral sclerosis; hiPSC; motor neuron disease; disease modeling

SUMMARY:

We describe a method for differentiating spinal cord human induced pluripotent-derived astrocytes and neurons and their co-culture for electrophysiological recording.

ABSTRACT:

Human pluripotent stem cell-derived astrocytes (hiPSC-A) and neurons (hiPSC-N) provide a powerful tool for modeling Amyotrophic Lateral Sclerosis (ALS) pathophysiology *in vitro*. Multi-electrode array (MEA) recordings are a means to record electrical field potentials from large populations of neurons and analyze network activity over time. It was previously demonstrated that the presence of hiPSC-A that are differentiated using techniques to promote a spinal cord astrocyte phenotype improved maturation and electrophysiological activity of regionally specific spinal cord hiPSC-motor neurons (MN) when compared to those cultured without hiPSC-A or in the presence of rodent astrocytes. Described here is a method to co-culture spinal cord hiPSC-A with hiPSC-MN and record electrophysiological activity using MEA recordings. While the differentiation protocols described here are particular to astrocytes and neurons that are regionally specific to the spinal cord, the co-culturing platform can be applied to astrocytes and

neurons differentiated with techniques specific to other fates, including cortical hiPSC-A and hiPSC-N. These protocols aim to provide an electrophysiological assay to inform about glia-neuron interactions and provide a platform for testing drugs with therapeutic potential in ALS.

INTRODUCTION:

Human pluripotent stem cell-derived astrocytes (hiPSC-A) and neurons (hiPSC-N) are powerful tools for modeling Amyotrophic Lateral Sclerosis (ALS) pathophysiology *in vitro* and provide a translational paradigm for drug discovery strategies[1]. Researchers have demonstrated that the co-culture of hiPSC-A with hiPSC-N enhances the morphological, molecular, electrophysiological, and pharmacological maturation of both cell types, generating complex neuronal networks and astrocyte-neuron interactions that resemble their *in vivo* counterparts[2,3]. Similar co-culture experiments can recapitulate hallmarks of ALS pathobiology such as astrocyte-mediated neurotoxicity[4,5] and neuronal hyper-excitability[6]. Additionally, with advancements in differentiation protocols, human induced pluripotent stem cells (hiPSC) can be differentiated into regionally-specific neural subtypes, including cortical and spinal cord hiPSC-A and hiPSC-N[7,8]. These strategies provide the potential for modeling cortical and spinal motor neuron pathology in ALS as well as the astrocytic influence on both. However, this requires that there is a reproducible functional assay to determine these effects.

Recently it was shown that multi-electrode array (MEA) recording is particularly suitable for the electrophysiological characterization of neuron-astrocyte co-cultures[2]. As opposed to single-cell electrophysiological analyses, these high-density electrode arrays passively record extracellular field potentials from large populations of neurons without disrupting culture conditions and preserving cell membranes' integrity. These platforms are particularly useful for recording the cellular and network activity of cultures over time and in response to pharmacologic manipulation. Finally, when the presence of astrocytes is a culture variable, MEA recordings can provide functional insights into astrocyte-neuron bidirectional interactions[2,9].

Presented here is an optimized protocol for the differentiation of hiPSC into spinal cord hiPSC-A and hiPSC-motor neurons (MN) that has been previously validated[2]. The spinal cord hiPSC-A differentiation protocol consistently results in astrocyte cultures, which are positive for S100 calcium-binding protein B (S100β), glial fibrillary acidic protein (GFAP), and Homeobox B4 (HOXB4) in up to 80%, 50%, and 90% of cells, respectively, indicating a maturing glial and spinal cord specification[2,10]. The hiPSC-MN differentiation protocol generates neurons that are >90% positive for choline acetyltransferase (ChAT), suggestive of a mature alpha-motor neuron identity[2]. Additionally, the protocol describes techniques for the generation of hiPSC-A/MN co-cultures previously demonstrated to result in neurons with enhanced morphological complexity by Scholl analysis and immunofluorescent microscopy when compared to neuronal cultures without astrocytes or with rodent astrocytes[2]. While these descriptions are specific to spinal cord hiPSC-A and hiPSC-N, a unique advantage is that the initial independent culture of astrocytes and neurons followed by steps to co-culture at later time points can be translated to study the effects of neuron-astrocyte interactions from other specific regions as well as disease-specific cells[7,8]. Finally, the protocol describes how to grow these cultures on MEA plates so that functional

activity as a factor of co-culture composition can be studied over time with the ability to manipulate cellular composition as well as culture conditions.

The goal of these protocols is to provide a functional assay to investigate astrocyte-neuron interactions, examine disease-specific changes, and test drugs with therapeutic potential in the field of ALS. Video instructions are provided for the most challenging steps of this protocol.

PROTOCOL:

1. Cell culture media preparation

1.1 Prepare the individual cell culture media using the compositions mentioned in **Table 1**.

1.2 Mix and sterile filter the media in 500 mL filtered bottles, and store protected from light at 4 °C for up to 2 weeks.

2. Maintaining and passaging non-confluent human induced Pluripotent Stem Cells (hiPSC)

2.1 Thaw the basement membrane matrix (stored in aliquots at -80 °C) in a 2–8 °C refrigerator overnight.

2.2 Dilute the basement membrane matrix at 1:100 (v/v) in cold phosphate-buffered saline (PBS) or Dulbecco's Modified Eagles Medium (DMEM).

2.3 Add enough basement membrane matrix to coat the bottom of the tissue culture plate (5 mL for a 10 cm plate, 2 mL for single wells of 6-well plate) and incubate at 37 °C for a minimum of 30 min overnight or at room temperature for a minimum of 1 h.

2.4 Aspirate the diluted basement membrane matrix and wash the plate once with PBS before adding culture media and seeding cells.

2.5 Examine the plates daily to determine and anticipate when they are ready to be passaged. Passage plates of hiPSC once the majority of colonies have become sufficiently dense so that the center of the colonies displays scattered white areas. Do not allow the plates to mature past this point. It will cause excess differentiation leading to the appearance of a yellow area at the center of the colonies due to cell multi-layering, the superabundance of elongated cells at the edges, loose compactness within colonies, or increased cell death.

2.6 On the day of passage, draw grid lines on the bottom outer surface of the plates with a marker to facilitate accurate coverage of the entire plate.

2.7 Detach differentiated colonies in a culture hood using a phase-contrast microscope (10x magnification) manually scraping with a 200 µL pipette tip.

2.8 Rinse the plates twice with PBS (5 mL per wash for 10 cm plates).

2.9 Add 5 mL of pre-warmed tissue dissociation protease (**Table of Materials**) and incubate the cells at 37 °C until the edges of the colonies start to round up (4–7 min), but prior to the colonies lifting from the plate.

2.10 Carefully aspirate the dissociation protease. Gently rinse the plate twice with PBS, being careful not to dislodge the colonies.

2.11 Add 5 mL of pre-warmed hiPSC medium (**Table 1**) to the culture plate.

2.12 Lift the colonies while breaking them up into smaller cell aggregates by scratching the entire plate with the tip of a 5 mL pipette using a back-and-forth motion from top to bottom.

2.13 Turn the plate 90° and repeat the above step.

2.14 Triturate the cell aggregates gently by pipetting up and down with a 5 mL pipette and check the size of the aggregates periodically under a microscope until the majority of the cell aggregates are approximately 50–100 cells.

2.15 Seed the cell aggregates into the pre-coated basement membrane matrix plates, using an additional hiPSC medium to wash the original plate. Collect and transfer most of the cell aggregates to the new plates using a 5 mL pipette.

NOTE: If the protocol is applied consistently, the original plate should contain iPSC colonies covering approximately 50% of the culture surface prior to passage. In this case, the split ratio should be from one plate to five new plates. However, this may need to be adjusted depending on the growth conditions and cell line-specific growth rates.

2.16 Shake the newly seeded plates back and forth to distribute the aggregates evenly.

2.17 Transfer the plates to an incubator (37 °C and 5% CO_2), and leave them undisturbed overnight to allow for proper cell adhesion.

2.18 Perform a complete medium change every day with 10 mL of hiPSC medium. If there is excess cellular debris on the day following passage, wash once with 5 mL of the medium prior to media change.

2.19 Plan to passage next when white areas appear at the center of the majority of the iPSC colonies (step 2.5). This will occur 4–6 days after each passage.

3. Freezing hiPSC

3.1 Perform the initial steps as in steps 2.5–2.14 for hiPSC passage.

3.2 Collect the cell aggregates into a 15 mL tube and spin at 200 x *g* for 2 min.

3.3 Aspirate the supernatant, resuspend the pellet in a freezing medium, and transfer to cryotubes using a 5 mL pipette.

NOTE: Similar to the passage steps, the usual split ratio is one iPSC plate to five cryotubes, depending on the density of colonies. Use a total volume of 1 mL of freezing medium for each cryotube.

3.4 Place the cryotubes into a chilled cryopreservation container and transfer it to -80 °C overnight.

3.5 Transfer the cells to liquid nitrogen after 24 h.

4. Thawing hiPSC

4.1 Coat a 10 cm plate with the basement membrane matrix (see 2.1–2.3).

4.2 Remove an iPSC cryotube from liquid nitrogen and place it immediately in a 37 °C water bath for up to 2 min to thaw quickly. Shake the vial in the bath until it is partially thawed and contains a small piece of ice surrounded by liquid.

4.3 Transfer the cell aggregates from the cryotube to a 15 mL conical tube using a 1000 µL pipette. Add up to 15 mL of pre-warmed hiPSC medium to dilute the dimethyl sulfoxide (DMSO) in the freezing media.

4.4 Centrifuge the 15 mL conical tube at 200 x *g* for 2 min.

4.5 Aspirate the supernatant and use a 5 mL pipette to resuspend the cell pellet in hiPSC medium containing 20 µM of Rho-associated coiled-coil forming protein serine/threonine kinase inhibitor (ROCK-I, compound Y-27632) to avoid further trituration of cell aggregates.

4.6 Transfer the cell aggregates to a basement membrane matrix-coated 10 cm plate using a 5 mL pipette.

NOTE: If the protocol is applied consistently, cells in one cryotube can be transferred to a single 10 cm plate.

4.7 Shake the plate to distribute the aggregates evenly.

4.8 Transfer the plate to an incubator and leave it undisturbed overnight to allow for proper cell adhesion.

4.9 Perform a complete medium change the day after thawing with 10 mL of hiPSC medium without ROCK-I.

5. Differentiating hiPSC into spinal cord Neural Progenitor Cells (NPC)

5.1 Ensure that the basement membrane matrix coated 6-well plates are ready to use prior to starting differentiation.

5.2 Start with iPSC that have been passaged at least once and preferably twice after thawing and with at least four 10 cm plates (see explanation in step 5.11).

5.3 Perform the initial steps as in steps 2.5–2.14 for the hiPSC passage.

5.4 Transfer the cell aggregates from at least two 10 cm plates into a 15 mL tube using a 5mL pipette.

5.5 Centrifuge at 200 x *g* for 2 min.

5.6 Aspirate the supernatant and resuspend the cell pellet with 10 mL of hiPSC medium using a 10 mL pipette, and then centrifuge again at 200 x *g* for 2 min to loosen the cell-to-cell adhesions in each aggregate.

5.7 Aspirate the supernatant and resuspend the cell pellet in 1 mL of hiPSC medium containing 20 µM ROCK-I using a 1000 µL pipette.

5.8 Pipette up and down with a 1000 µL pipette to triturate the cell aggregates. Check the size of the aggregates under a microscope periodically until the majority of the cell suspension comprises single cells or small aggregates of <10 cells.

5.9 Count the cells with a hemocytometer (preferred) or an automated cell counter and calculate the cell density in the cell suspension.

5.10 Plate 3.0 x 10^6 cells in each well of a 6-well plate pre-coated with basement membrane matrix. Use a 1000 µL pipette to transfer the cell suspension.

NOTE: If the protocol is applied consistently, this corresponds to a ratio of two 10 cm plates into a single well of a 6-well/plate.

5.11 Repeat steps 5.3–5.10 with a second batch of two 10 cm plates and plate the final product into a basement membrane matrix coated well of a second 6-well plate. Use a 1000 µL pipette to transfer the cell suspension.

NOTE: Ideal completion of the spinal cord NPC protocol requires two wells equal to four 10 cm plates. For ease of workflow, complete steps 5.3– 5.10 with two batches of two plates, each with the final product being two separate 6-well plates and each with one well containing 3.0 x 10^6 cells in it.

5.12 Maintain the resulting human iPSC monolayer in hiPSC medium containing 20 µM ROCK-I until the confluency reaches >90%, typically on the following day but no longer than 3 days after initial plating to avoid spontaneous (uncontrolled) differentiation.

5.13 If differentiation cannot be started on the day following cell plating, wash the cells once with PBS and change media daily to a fresh hiPSC medium containing 20 µM ROCK-I. If it does not confluence >90% within 3 days of plating, discard the cells and start the protocol over.

5.14 Once >90% confluence is reached, start differentiation. This is day *in vitro* 1 (DIV1) of the differentiation protocol.

5.15 On DIV 1, discard the hiPSC medium containing 20 µM ROCK-I and rinse twice with PBS to remove the fetal growth factor (FGF) and transforming growth factor-beta (TGFβ) present in the stem cell medium.

5.16 Change the medium to WiCell medium (**Table 1**) supplemented with 0.2 µM of LDN193189 (LDN) and 10 µM of SB431542 (SB) for 48 h.

5.17 On DIV 3, wash once with PBS and change the medium to WiCell medium supplemented with LDN (0.5 µM) + SB (10 µM) + Retinoic Acid (RA; 1µM) for 48 h.

5.18 On DIV 5, wash once with PBS and change the media to WiCell: Neural induction medium (NIM) base (**Table 1**) (50%:50%, v/v) supplemented with LDN (0.5 µM) + SB (10 µM) + RA (1 µM) for 48 h.

5.19 On DIV 7, perform medium exchange with the same medium as in step 5.18.

5.20 On DIV 8, wash once with PBS and change medium to WiCell:NIM base (50%:50%) supplemented with RA (1 µM) + puromorphamine (PMN) (1 µM) + Recombinant human-brain-derived neurotrophic factor (BDNF) (10 ng/mL) + Ascorbic acid (ASAC) (0.4 µg/mL) for 48 h.

5.21 On DIV 10, wash once with PBS and change medium to NIM base (100%) supplemented as mentioned in step 5.20 for 48 h.

5.22 On DIV 11 or 12, coat a 25 cm^2 sterile culture flask with basement membrane matrix in preparation for passage.

5.23 Aspirate the medium from each well of the 6-well plate l and rinse the cells once with PBS.

5.24 Add 2 mL of 0.05% trypsin to each well and incubate at 37 °C for 5–15 min; intermittently shaking the plates may help in cell detachment.

5.25 If the cells are still not in suspension, detach mechanically by ejecting NIM media from a 1000 µL pipette tip onto the cells with a circular motion (make sure to cover the whole surface), or by scraping with a cell scraper (not preferred).

5.26 Transfer the cells from 2 wells to a 15 mL tube and add trypsin inhibitor in appropriate proportion to the amount of trypsin used using a 5 mL pipette.

5.27 Centrifuge at 200 x *g* for 2 min, aspirate the supernatant, and then resuspend with 10 mL of NIM base using a 10 mL pipette.

5.28 Centrifuge again at 200 x *g* for 2 min to loosen the cell-to-cell adhesions.

5.29 After the second centrifugation step, remove the supernatant, and re-suspend the pellet with 1 mL of NIM medium (100%) supplemented with RA (1 µM) + PMN (1 µM) + BDNF (10 ng/mL) + ASAC (0.4 µg/mL) and ROCK-I (20 µM). Using a 1000 µL pipette tip, triturate the cells up and down approximately five times until a cloudy suspension is obtained.

5.30 Re-seed the aggregates in this medium to the basement membrane matrix coated 25 cm^2 sterile culture flask so that the final result is the combination of the cells from two wells of a 6 well plate to a single basement membrane matrix-coated 25 cm^2 sterile culture flask (2:1 passage). Use a 1000 µL pipette to transfer the cell suspension.

NOTE: The cells should be >90% confluent on DIV 13, and no further steps are needed until Day 14. If the cells are not confluent, keep ROCK-I in the media for an additional 1 or 2 days.

5.31 On DIV 14, change the medium to fresh NIM media + RA (1 µM) + PMN (1 µM) + BDNF (10 ng/mL) + ASAC (0.4 µg/mL).

5.32 On DIV 15, change the medium to NIM: Neural differentiation medium (NDM) base (**Table 1**) (50%:50%) with RA (1 µM) + PMN (1 µM) + ASAC (0.4 µg/mL) + Supplement B (50x) + BDNF (10 ng/mL) + Glial cell line-derived neurotrophic (GDNF) (10 ng/mL) + Insulin-like growth factor 1 (IGF-1) (10 ng/mL) + Ciliary neurotrophic factor (CNTF) (10 ng/mL). Change with fresh media every other day.

5.33 On DIV 21, change the medium to NDM base (100%) supplemented as in step 5.32, and change with fresh medium every other day.

5.34 On DIV 25–30, collect the NPC and either freeze them or passage them to a 10 cm plate for terminal differentiation.

5.35 Rinse the T-25 flask with PBS and add 0.05% trypsin.

5.36 Incubate for 5 min at 37 °C.

5.37 Rinse the trypsin and the cells with the NDM base and transfer to a 15 mL conical tube. Add the trypsin inhibitor in appropriate proportion to trypsin and centrifuge at 300 x *g* for 5 min.

5.38 Resuspend the cell pellet with motor neuron or astrocyte differentiation medium (**Table 1**) and use a 1000 µL pipette to triturate up and down to obtain a single cell suspension (usually up to 5 times).

5.39 Count the cells and transfer to a 10 cm plate coated as described in section 6, using either differentiation media +Rock-I, to differentiate hiPSC-MN and hiPSC-A.

5.40 Alternatively, freeze the cultures by resuspending in a freezing medium composed of 90% NDM medium with growth factors (as in steps 5.32–5.33) and 10% DMSO, triturate similarly, and then transfer to a cryotube. Aliquot 6 x 10^6 NPC per cryotube.

6. Thawing NPC cultures

6.1 Coat 10 cm plates with polyornithine (PLO) and laminin the day prior to thawing the cells.

6.1.1 Add 5 mL of PLO diluted to 100 µg/mL in PBS or distilled water (dH_2O) to plates. Incubate at 37 °C for a minimum of 1 h up to overnight.

6.1.2 Aspirate the PLO solution and rinse the plates three times with PBS. (PLO is toxic if not properly washed.)

6.1.3 Add laminin diluted in PBS to a concentration of 10 µg/mL to the PLO coated plates. Incubate at 37 °C for a minimum of 1 h, preferably overnight. After incubation, aspirate the laminin solution without rinsing to enhance cell adhesion.

NOTE: The plates can be coated with PLO ahead of time, washed three times with water, dried in a sterile hood, and stored at 4 °C.

6.2 Thaw one NPC vial (i.e., DIV 25 or 30) containing 6 x 10^6 cells/vial for each 10 cm cell culture plate.

6.3 Remove the NPC cryotube from liquid nitrogen and place immediately in a 37 °C water bath for up to 2 min. Quickly thaw by shaking the vial until there is a small ice piece surrounded by liquid.

6.4 Transfer cell aggregates into a 15 mL conical using a 1000 µL pipette and add up to 15 mL of pre-warmed NDM or Dulbecco's Modified Eagles Medium (DMEM) / Ham's F21 F12 supplement to dilute the DMSO.

6.5 Centrifuge at 300 x *g* for 5 min.

6.6 Aspirate the supernatant, resuspend the cell pellet with either astrocyte or MN differentiation medium (**Table 1**) + ROCK-I (20 µM) and use a 1000 µL pipette to triturate up and down until a single cell suspension is obtained (up to 5 times).

6.7 Transfer the single-cell suspension to a PLO-Laminin coated 10 cm plate.

6.8 Transfer the plate to an incubator and leave it undisturbed overnight to allow for proper cell adhesion.

7. Differentiating spinal cord NPC into motor neurons

7.1 Perform initial steps as mentioned in steps 6.1–6.8, with the final medium being MN differentiation medium + ROCK-I (20 µM).

7.2 The day after plating, perform a complete exchange of the medium with MN differentiation medium without ROCK-I, and then change the media every other day. Over the weekend, feed cells on Friday and then again on Monday. Rinse the cells with PBS when needed to remove cell debris.

7.3 Change the medium with MN differentiation medium + cytosine arabinoside (ARA-C) (0.02 µM) and incubate for 48 h when glial committed progenitors emerge as single proliferating flat cells under post-mitotic MN progenitors aggregated in cell clusters.

NOTE: Usually, this occurs within the first 7 days after initial plating, although there may be variability depending on the cell line.

7.4 After 48 h, aspirate the medium containing ARA-C, rinse cultures gently with PBS three times, and change the medium to fresh MN medium without ARA-C.

7.5 After treatment with ARA-C, perform medium exchanges every other day (or Monday, Wednesday, and Friday) by removing the old medium with a manual pipette rather than a vacuum aspirator to prevent premature detachment of neuronal clusters. In addition, enrich the

MN medium with Laminin 1 µg/mL once a week to further enhance neuronal attachment to the culture plates.

8. Differentiating NPC into spinal cord astrocytes

8.1 Thaw NPC cultures as in section 6, using Astrocyte Differentiation Medium (**Table 1**) with the addition of fetal bovine serum (FBS) with a volume for volume concentration of 1% (NS + 1% FBS) as well as ROCK-I (20 µM) for plating.

NOTE: Commercially available serum replacement (**Table 1**) can be substituted for FBS using the same dilution factors.

8.2 On the day after plating, change the culture medium with NS + 1% FBS medium without ROCK-inhibitor, and then change the medium every other day. Over weekends, feed cells on Friday and then again on Monday. Rinse the cells with PBS when needed to remove cell debris. If the cells continue to die, supplement media with ROCK-I (10 µM) to promote cell survival. Be cautious not to use ROCK-I too often or too long, given the potential to select immortal cells.

8.3 Allow the astrocytes to become confluent before passaging them.

NOTE: The cell size will increase over time, so there is no set number to passage. The typical passaging ratio is 1:2 or 1:3. In the case of poor survival or passaging to too many plates, combine two (or more, as needed) 10 cm plates to one 10 cm plate to maintain confluency.

8.4 To passage astrocyte progenitor cultures, wash the plates once with PBS (to remove FBS), incubate with 0.05% trypsin for 5 min, collect the cells in NS medium + 1% FBS (FBS will inactivate trypsin), and then centrifuge at 300 x *g* for 5 min. Aspirate the supernatant, resuspend the cells in NS medium + 1% FBS, and then triturate to a single-cell suspension. Distribute cells in NS + 1% FBS to new 10 cm plates.

NOTE: In addition to expanding astrocyte cultures, repeated passaging of plates serves the purpose of killing any remaining progenitor cells that have chosen a neuronal fate. Therefore, even if there is not a very high mitotic rate, plates can be passaged at a 1:1 ratio if there appears to be neuronal contamination.

8.5 Change the coating over the course of differentiation to enhance the yield as follows.

8.5.1 Plate the initially thawed NPC cultures and the first passages after initial plating onto PLO/Laminin.

8.5.2 Plate the immature astrocytes on basement membrane matrix coated plates.

8.5.3 Plate more mature astrocytes (i.e., after day 90) on either basement membrane matrix, PLO/Laminin coated plates (typically for co-culture with neurons), or on uncoated plates.

8.6 Freeze the astrocyte progenitors at any time point over the 60-day maturation period to synchronize cultures or save for later experiments. When thawing beyond the NPC stage, thaw the astrocyte progenitors onto the basement membrane matrix coated plates rather than PLO/Laminin.

8.7 At DIV 90 and thereafter, switch the medium from NS + 1% FBS to NS + 5% FBS to promote astrocyte survival and decrease their proliferation.

9. Co-culturing MN and spinal cord astrocytes in multi-electrode array plates

9.1 Differentiate and plate the motor neurons and astrocytes when they are ready to be used on the same day and aged to DIV 60 for the motor neurons and DIV 90 for the astrocytes.

9.2 Coat the MEA plates on the day before or on the day of plating as follows if working with 24-well plastic plates (**Table of Materials**).

9.3 Dilute PLO in water or PBS to 100 µg/mL.

9.3.1 Add 15–20 µL to each well (dependent on pipette comfort level), forming a droplet on the center of the well covering the area of the electrodes and surrounding area but not the entirety of the well.

9.3.2 Take care not to damage electrodes with the pipette tip. Be consistent with volume from well to well to ensure coverage of the same surface area in each well.

9.3.3 Incubate PLO at 37 °C for a minimum of 1 h (preferably 2 h).

NOTE: The small volumes will dry up if there is not sufficient humidity in the plates. Add water to the compartments surrounding wells to ensure sufficient humidity throughout the course of coating and recordings.

9.4 Aspirate as much PLO as possible using a plastic micropipette tip. Take care not to touch the electrodes. Wash with 250 µL of water three times. If using a vacuum aspirator for washes, do not allow the tip near the electrode array. After the third wash, remove as much water as possible, using a pipette tip as necessary. Let the surface dry under the cell culture hood with the lid removed.

9.5 Once plate surfaces are dry, add Laminin diluted to 10 µg/mL in PBS. Use 15–20 µL to cover each electrode array. Add water to the humidity compartments, replace the lid, and return the plate to 37 °C incubation for a minimum of 2 h up to overnight.

9.6 On the day of plating, rinse the MN and astrocyte cultures once with PBS and add trypsin 0.05% at 37 °C to lift cells (5 min). Collect into a 15 mL conical tube containing trypsin inhibitor and wash plates with medium or base to ensure that all the cells are collected. Centrifuge at 300 x *g* for 5 min and resuspend with a 1000 µL pipette to generate 1 mL of a single cell suspension. When re-suspending MN and astrocytes, switch to the co-culture medium (**Table 1**), with the addition of 20 µM ROCK-I.

9.7 Count the MN and astrocytes in parallel using a hemocytometer. While counting and making calculations, cap the cell suspensions and place them in a Styrofoam rack at 4 °C.

9.8 Calculate the volume required to resuspend the cultures to a concentration of 5 x 10^4 cells per 5 µL for motor neurons and 2.5 x 10^4 cells per 5 µL for astrocytes. Centrifuge the 1 mL cell suspensions at 300 x *g* for 5 min and resuspend in the calculated volume.

9.9 Calculate the number of desired wells to be seeded and multiply it by 5 µL of each cell suspension. Combine the required volume of neuron and astrocyte suspensions at a 1:1 ratio and mix by pipetting until thoroughly combined (usually twice), but avoid being too aggressive.

9.10 Remove the Laminin from each well of the MEA plate using a pipette tip. Transfer 10 µL of the final combined cell suspension to each well, forming a small droplet covering the electrode array to provide a cell density of 5 x 10^4 MN and 2.5 x 10^4 astrocytes per well.

NOTE: High cell density in the combined suspension requires frequent resuspension in between wells during the seeding step to ensure accurate and consistent cell counts.

9.11 Return the plates to the incubator for 20–30 min. Take care not to disturb the cell droplet and allow the cells to form initial attachments on the plates.

9.12 After 20–30 min, add warm co-culture media + ROCK-I to each well by pipetting 250 µL down on the wall of each well, followed by an additional 250 µL down the wall of the same well on the opposite side. Return the plate to the incubator.

9.13 Examine plates the day after seeding (Co-culture Day 1). If there is significant cell debris or dead cells, exchange the medium with a fresh co-culture medium containing ROCK-I. Otherwise, change the medium on the second day after seeding (Co-Culture Day 2) to the co-culture medium without ROCK-I. Perform half-medium exchanges (aspirate 50% and add 60% of the final volume to account for evaporation) twice a week.

9.14 If using MEA systems with single well 30 mm glass plates (**Table of Materials**), the plate preparation may take 2 or more days. Follow the steps below to perform this.

9.15 Lift the cells and cell debris from the previous MEA cultures with 0.05% Trypsin for 5–15

min.

9.16 Aspirate trypsin and rinse three times with water.

9.17 Sterilize by adding 70% ethanol and incubate in the hood for 10 min. Aspirate the ethanol, and then rinse with water three times.

9.18 Apply 1% anionic detergent with protease enzyme in water. Cover the plates with a lid and wrap with thermoplastic film and aluminum foil, and then leave them on a rocker at room temperature overnight.

9.19 Aspirate the detergent, and then rinse three times with water.

9.20 If planning to store the plates, add a sufficient volume of water. Cover the plates with a lid and wrap them with thermoplastic film and aluminum foil, and leave them in the refrigerator until next use.

9.21 If planning to coat, let the surface dry under the cell culture hood.

9.22 If extra sterilization is needed (e.g., previous infection or non-recent use), at this point only, expose MEA plates to ultraviolet (UV) light under the hood for 30–60 min.

NOTE: Avoiding frequent UV light will prevent damage to the electrodes. The use of UV light when plates have serum on them will result in nonfunctional electrodes, which is why it should only be used on cleaned plates.

9.23 When the plates are completely dry, proceed with plasma cleaning for 1 min to charge the glass surface of MEA plates and enhance coating effectiveness. Plasma clean at least once every 4–5 cycles of MEA plate cleaning-plating cycles.

9.23.1 As an alternative, when plasma cleaning is not feasible or not warranted, add a sufficient volume of FBS-containing medium to cover the MEA plates' surface and return them to the incubator overnight.

9.24 Use PLO/Laminin coating as described above in section 6. Add PLO solution (100 µg in PBS) immediately after plasma cleaning or aspirating and rinsing out the FBS-containing medium.

9.25 Plate the cells as above at the following densities: 1 x 10^5 hiPSC-A / plate and 5 x 10^5 hiPSC-MN/plate.

10 Multi-electrode array recording

10.1 Extract the raw voltage data at a 12.5 kHz sampling frequency, using a Butterworth filter

with a 200 Hz high pass and 3 kHz low pass filter. Set spike recognition as instantaneous time points of voltages ≥6 standard deviations from baseline. Identify bursts as an activity with >5 spikes in 100 ms. Define network activity when over 35% of total active electrodes fire within 100 ms with a minimum of 50 spikes per network burst.

10.2 Start recording as soon as possible after Co-Culture (CC) Day 1.

NOTE: Electrical activity will be rarely noted before CC Day 3.

10.3 Plate parallel cultures at similar densities and replicates in the appropriate culture plates or coverslips for biochemical assays, immunocytochemistry (ICC), or qPCR.

10.4 Perform recordings with the temperature set to 37 °C ad CO_2 at 5%. Transfer plates to the machine and allow them to equilibrate for at least 5 min prior to recording.

10.5 Record baseline activity either every other day or weekly, over 1–15 min depending on the experimental design. Do not record for at least 1 h after a medium exchange, and ideally wait for 24 h after every media exchange.

10.6 To prevent contamination, change the medium (i.e., half medium exchange as in step 9.14) after any recording in which the lid of the sterile plate has to be opened outside of the sterile hood (i.e., the application of drug compounds). Perform complete medium exchanges and washes to wash out drugs if plates are going to continue to be used beyond drug treatment.

10.7 For analysis purposes, use at least three technical and biological replicates for each condition. Express electrophysiological data as means of n ≥ 3 replicates.

11 Pharmacological assays on multi-electrode array

11.1 Use a different combination of co-cultures depending on the experimental question: ALS neurons with control astrocytes, control neurons with ALS astrocytes, ALS neurons and astrocytes, control neurons and astrocytes.

11.2 When investigating transient electrophysiological effects of compounds targeting either neurons or astrocytes, record baseline activity for a minimum of 1 min with plate lid removed but machine lid closed.

11.3 Manually open the lid to the machine without stopping the electrophysiological recording and exchange 25 µL of medium with the appropriate drug vehicle (usually fresh medium) using a multichannel pipette if treating multiple wells. Close the lid manually.

11.4 Record for an additional 1 min (or more if there are significant vehicle-induced changes from which the culture needs to recover).

11.5 Manually open the lid again and exchange 25 µL of medium with the drug of interest in the same vehicle. Close the lid to the machine and continue recording.

NOTE: The duration of recording and volume of drug/vehicle administered may vary or need to be optimized depending on the experimental question.

11.6 For analysis purposes, ensure that the addition of the vehicle does not provoke significant changes in electrophysiological parameters. Calculate percentage changes of the activity after drug compared to after vehicle addition and compare among conditions.

11.7 To investigate longitudinal electrophysiological effects, such as candidate disease-modifying drugs, record baseline activity outlined in step 11.2. For this, use either the co-culture medium containing the drug(s) of interest in the appropriate vehicle or the medium containing the vehicle only.

11.8 For analysis purposes, compare the time-point recordings from ALS or control co-cultures longitudinally treated with drugs to each other and control conditions.

REPRESENTATIVE RESULTS:

The spinal cord patterning protocol for the generation of hiPSC-MN and spinal cord hiPSC-A is outlined in **Figure 1**. In this protocol, hiPSCs are maintained and passaged as non-confluent colonies (**Figure 2A**). Neurogenesis is initiated (neural induction) through dual SMAD inhibition by the addition of LDN193189 and SB431542, inactivating the bone morphogenetic protein (BMP) and the transforming growth factor-beta (TGF-β) pathways, respectively. A monolayer-based method is used for this step where hiPSC are plated onto an adherent matrix, i.e., basement membrane matrix, to generate a neuroepithelium-like 2D culture. Morphologically, neural induction is marked by the transition from iPSC with large nuclei and round shape to neuroepithelial (stem) cells that are tightly compacted, with a large cytoplasm and cylindrical shape. These cells will divide horizontally as well as vertically, generating a multilayered epithelium (**Figure 2B**,**i**).

The preference for a 2D, as opposed to a 3D strategy (i.e., embryoid body-based), relies on literature evidence[11,12] suggesting that the former may promote spinal cord patterning by introducing cell-extrinsic environmental cues[13]. Neuroepithelial cells are then temporally and spatially patterned to generate region-specific, i.e., spinal cord, neural progenitor cells (NPC) (**Figure 1**). Two key morphogens are used for this purpose: retinoic acid, which determines caudalization, and purmorphamine, a hedgehog signaling agonist, which causes ventral specification. In their absence, the cell-intrinsic regional identity of NPC would be rostral and dorsal[14–16].

From DIV 15 to DIV 25–DIV 30, the regional identity of these NPC is reinforced by supplementing media with the morphogens. At the same time, the early exposure to neuronal growth factors

and a gliogenic cytokine such as ciliary neurotrophic factor (CNTF) generates a mixed population of NPC (**Figure 2B**,**ii**). Morphologically these cells are less compacted than neuroepithelial cells, displaying few short processes, arranged in a monolayer after disruption of the columnar epithelium after passaging at DIV 12. At a closer view, some of these cells are elongated, while others display multiple processes, which indicates early commitment toward a glial vs. neuronal fate, respectively.

Neurons will emerge spontaneously from this mixed population unless the gliogenic switch is activated (**Figure 1** and **Figure 2B**,**iii**). The addition of the Notch pathway inhibitor, Compound E, will enhance lower MN differentiation[17]. The spinal cord identity of these cells is supported by high levels of ChAT expression (**Figure 2C**,**i**). Additionally, it has been previously shown[2] that a subpopulation of these motor neurons is also ISL1+.

Astrocyte differentiation is induced by the gliogenic switch through activation of JAK/STAT pathway (**Figure 1** and Figure **2B**,**iv**). CNTF and likely other cytokines contained in fetal bovine serum serve this purpose in the proposed protocol. Time is an essential factor as well, since gliogenesis will spontaneously follow after neuronogenesis due to cell-intrinsic clues. After DIV 90, hiPSC-A displays a maturing phenotype as indicated by S100β and GFAP expression[2,11], while their spinal cord regional identity is supported by >90% expression of HOXB4 (**Figure 2C**, **ii**)[2,11,14].

The method for co-culturing hiPSC-MN and hiPSC-A is notable for simultaneous plating of these cell subtypes as opposed to techniques where neurons are serially plated on the top of astrocyte cultures. In these simultaneous co-cultures, cells will rearrange spontaneously, with astrocytes creating a feeding layer at the bottom and neurons connecting in networks at the top (**Figure 2C**,**iii**). This strategy has previously shown[2] to allow for a more uniform distribution of neurons than other co-culture strategies, where these cell types tend to cluster.

Human iPSC-MN are plated alone or in co-culture with hiPSC-A on a 24-well MEA plate (n = 12 per condition), with each well containing 16 electrodes (**Figure 3A**). Phase-contrast images of either mono- or co-cultures and raster plots of spiking activity from two representative wells are shown (**Figure 3B,C**). Human iPSC-A enhances the electrophysiological maturation of hiPSC-MN, as shown by significantly higher degrees of spiking and bursting activity in the co-cultures (**Figure 3D**). This parallels the effects of hiPSC-A on the morphological and molecular maturation of hiPSC-MN that has been previously demonstrated[2].

Some of the technical challenges of this protocol are detailed in the video instructions, where the techniques for plating and recording using MEA platforms as well as representative recordings from these cultures, are shown.

FIGURE LEGENDS:

Figure 1: Protocol for the generation of spinal cord hiPSC-MN and hiPSC-A and their co-culture. (**A**) Timeline of the differentiation protocol detailing critical stages. In the insert, focus on the 30-day protocol for generating spinal cord NPC, with day-by-day actions (E exchange medium, P

passage, or no action), cell culture base, and supplements (for additional information on the composition, see **Table 1** and **Table of Materials**). (**B**) Lineages and cell fate commitment are schematically represented. Co-cultures are generated when mature hiPSC-MN (i.e., DIV 60) and hiPSC-A (i.e., DIV 90) are mixed and plated simultaneously. (Illustration created with BioRender).

Figure 2: Morphological changes during the spinal cord differentiation protocol. (**A**) Phase contrast microscope images of hiPSC. Panels (**i**) (lower power, 10x) and (**ii**) (higher power, 20x) show normal hiPSC, panels (**iii**) (10x) and (**iv**) (20x) show a differentiated hiPSC colony. Scale bars = 200 µm and 50 µm. (**B**) Representative phase contrast microscope images of neuroepithelial cells at DIV 5 (**i**), NPCs at DIV 21 (**ii**), and mature DIV 60 motor neurons (**iii**) and DIV 90 astrocytes (**iv**). Scale bars = 100 µm and 50 µm (**C**) Representative immunocytochemistry images on 40x oil of hiPSC-MN (**i**), hiPSC-A (**ii**) and their co-culture (**iii**). Maturation and region-specific markers were targeted. Scale bar = 20 µm.

Figure 3: Multi-electrode array recording of hiPSC-MN. (**A**) Heat map of mean spiking activity from a single MEA plate (n = 24 wells) at DIV 18 after plating. Wells in rows A and B (n = 12 wells / technical replicates) represent cultures of hiPSC-MN alone, while wells in rows C and D (n = 12 wells) are co-cultures of hiPSC-MN/hiPSC-A. (**B**) Representative phase-contrast images of hiPSC-MN alone (MN) and in co-culture with hiPSC-A (MN+SCA) on MEA plates. Neurons aggregate in large cell clusters when cultured alone, while the co-culture of hiPSC-MN with hiPSC-A results in evenly distributed monolayers. Scale bar = 50 µm. (**C**) Representative raster plot of spiking activity over 120 s recording time from a single well with hiPSC-MN alone and hiPSC-MN in co-culture with hiPSC-A. Network burst activity across all electrodes is highlighted with a purple box. (**D**) Quantification and comparison of spiking and bursting activity between neurons alone and neurons in co-culture with astrocytes from the MEA plate shown in panel A. (*** $p < 0.001$, **** $p < 0.0001$).

DISCUSSION:

To date, hiPSC- and MEA-based methods for electrophysiological recordings of astrocyte-neuron co-cultures have found limited application in the field of ALS[6] and still not in fully human platforms, in contrast to their more widespread use for *in vitro* modeling of epilepsy[9]. This platform, however, has the potential to address pathophysiologically relevant questions in ALS research, such as the mechanisms of neuronal hyperexcitability, astrocyte contribution to neurotoxicity, or the role of network activity in the progression of the disease. Additionally, this platform allows for the collection of prospective electrophysiological data for over 9 months *in vitro* and, therefore, provides an approach for testing compounds with therapeutic potential[2].

Protocols for the generation of hiPSC-N and hiPSC-A found in the literature differ extensively in relation to media conditions, the timing of cultures, yield, and maturation profiles, among other factors. A major advantage of the proposed platform is that astrocytes and neurons are differentiated separately and cultured together at later time points by simultaneously plating the two cell types. After optimization for cell densities and timing, this method can be translated to be used with cell types derived from other protocols as well, including other region-specific cells.

Paramount to the proposed platform is the regional specification of motor neurons and astrocytes. While the proposed protocol is spinal cord specific in its ability to generate $ChAT^+$ MN and $HOXB4^+$ astrocytes, well-established differentiation techniques can be used to generate hiPSC-derived neurons and astrocytes displaying cortical identities, such as $CTIP2^+$ layer V cortical motor neurons[18] and $OTX2^+$ forebrain astrocytes[14]. Thus, the proposed platform has the potential to model neural circuits of the cortex and the spinal cord, as well as their connections.

Multiple systems are available for MEA recording. Plastic 24-well MEA plates with 16 electrodes per well with CO_2 and temperature control were preferred for the study. This platform is particularly suitable for high-throughput screening as opposed to systems based on single well recordings. The main limitation of plastic plates is that the surface may be more susceptible to degradation over repeated uses. Systems based on glass MEA plates have the advantage of being reusable multiple times after appropriate treatments as outlined above (up 20 to times), without significant loss of data recording quality. However, these treatments and the coating methods are more time-consuming and technically challenging, given the hydrophobic surface of these plates.

One of the main obstacles of iPSC- and MEA-based methods for electrophysiological recording is the variability and reproducibility of experimental findings. Previous studies show that MEA activity of neurons is dependent upon maturation that is influenced by multiple factors, including, but not limited to, appropriate differentiation techniques used in the generation of both astrocytes and neurons, cell density of neurons, the ratio of astrocytes to neurons throughout differentiation and maturation, sequence of astrocyte and neuron co-cultures[2]. Standardizing these variables as proposed in this protocol is one way to ensure reproducibility. The choice of multiwell MEA systems generating high throughput data will account for experimental variability. If the electrophysiological activity of a culture is less than what is expected at a given time point, it is important to determine that successful differentiation has occurred and sister cultures that can be immunocytochemically or biochemically analyzed are helpful. Given that a very small volume of cells is seeded initially, small pipetting errors can result in relatively large changes in cell numbers and, therefore, density. Using MEA plates that allow for direct visualization and assessment of cell density is important as well.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGMENTS:

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Figure 1

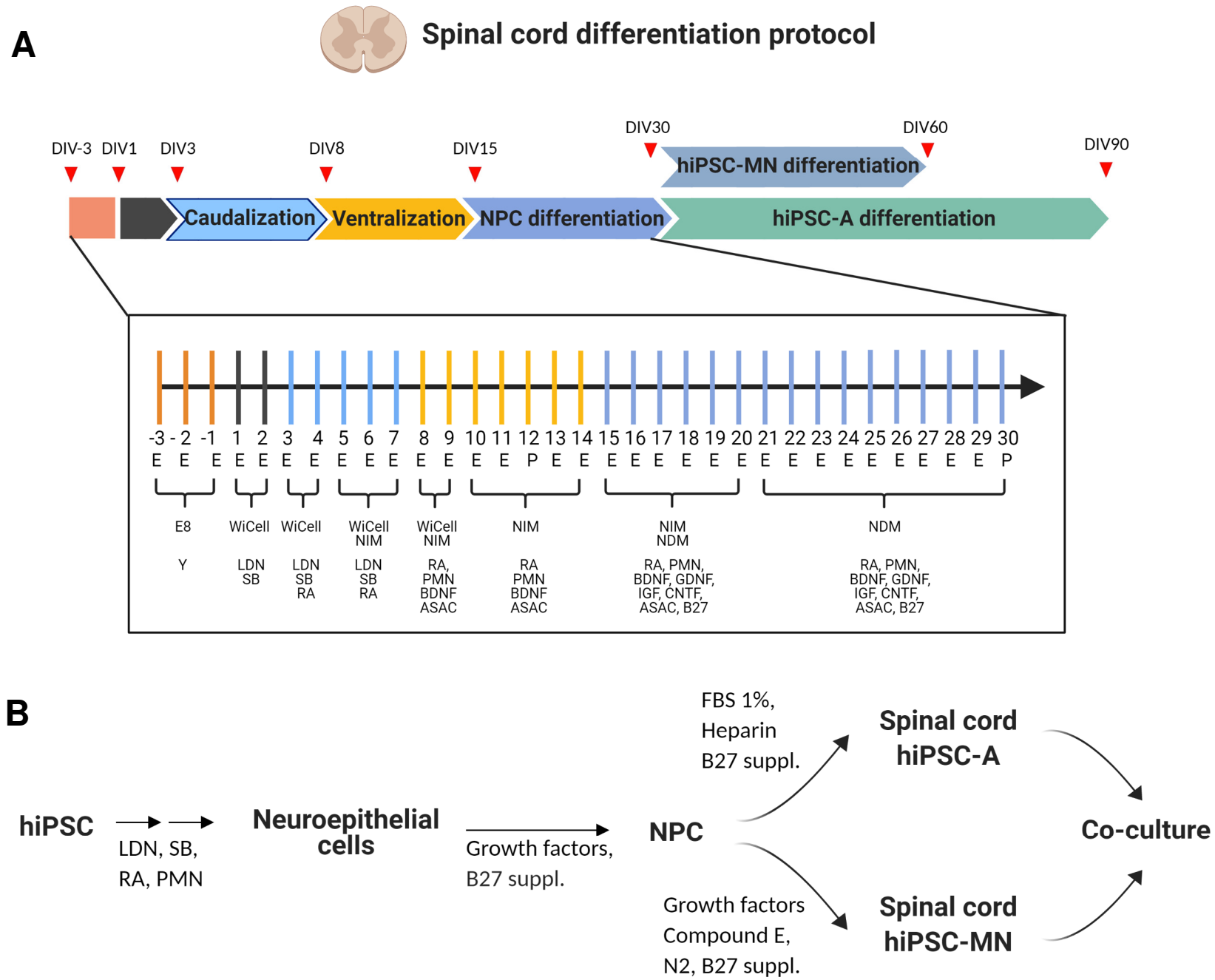

Figure 1

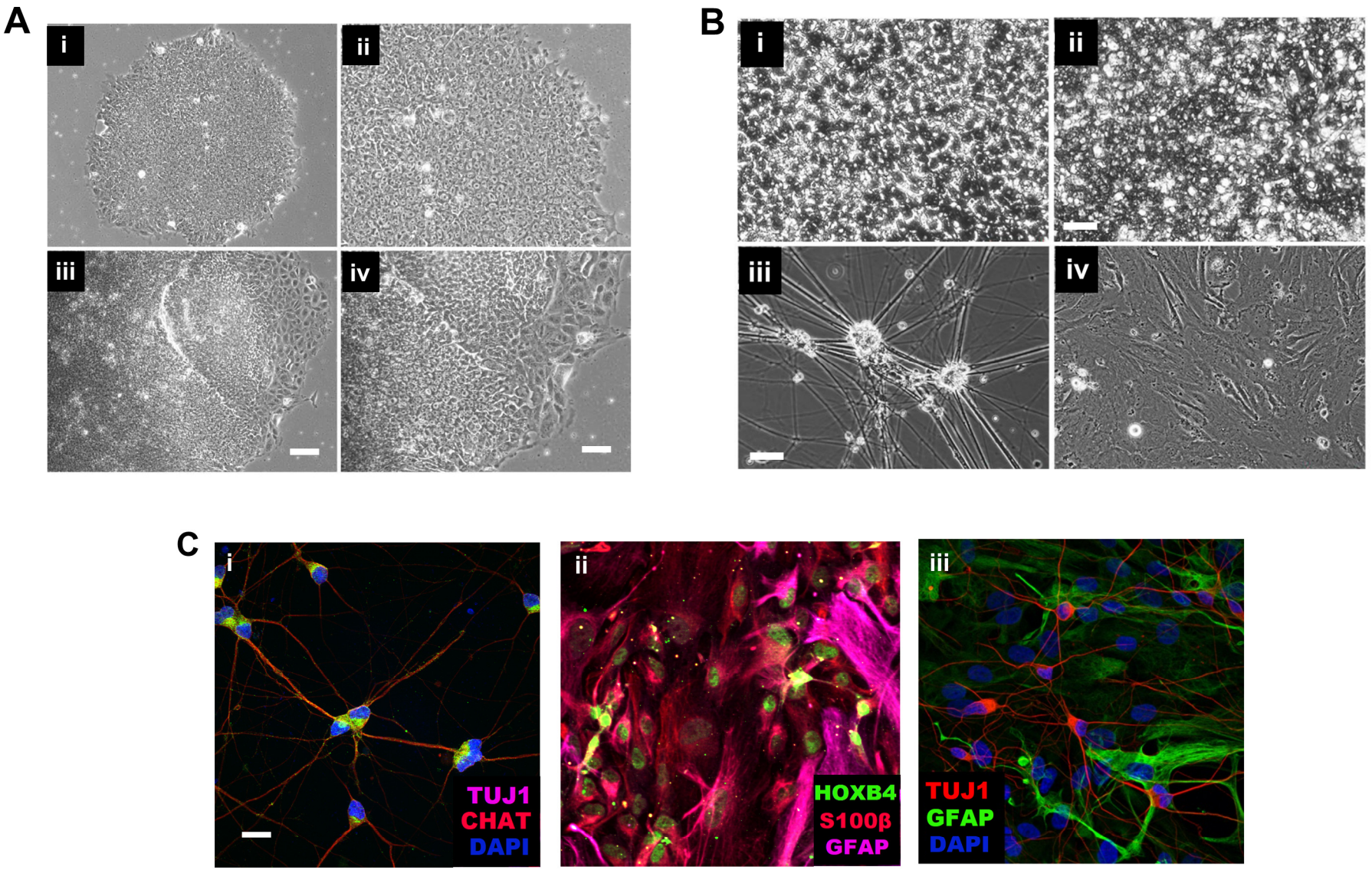

Figure 2

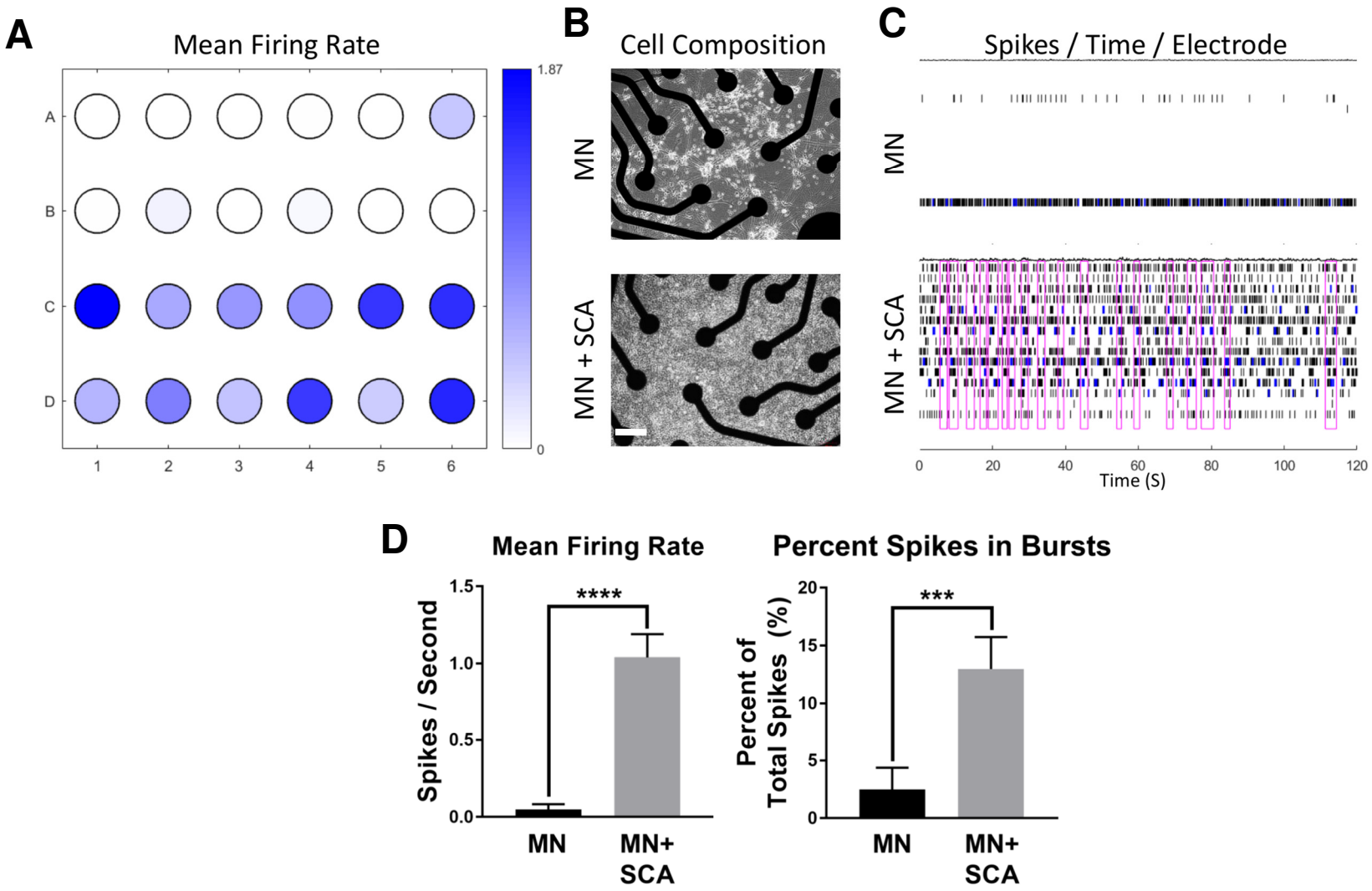

Figure 3

Culture Media

Medium name	Reagents
"WiCell" medium	DMEM/F12 L-Glutamine NEAA β-ME KSR
Neural induction Medium (NIM)	DMEM/F12 L-Glutamine NEAA N2 Penicillin/streptomycin Heparin
Neural Differentiation Medium (NDM)	Neurobasal L-Glutamine NEAA N2 Penicillin/streptomycin
Astrocyte Differentiation Medium ("NS") with FBS	DMEM/F12 L-Glutamine NEAA B27 Penicillin/streptomycin Heparin FBS
Motor neuron differentiation medium	NDM ASAC PMN BDNF CNTF IGF-1 GDNF RA

	Compound E B27
Co-culture medium	Motor neuron differentiation medium Laminin Amphotericin B FBS
hipsc- Medium	Essential 8 medium Essential 8 Supplement

Concentrations

1x
100x
100x
110 µM
20% (v/v)
1x
100x
100x
100x
100x
2 µg/mL
1x
100x
100x
100x
100x
1x
100x
100x
50x
100x
2 µg/mL
1% to 5% (v/v)
1x
0.4 µg/mL
1 µM
10 ng/mL
10 ng/mL
10 ng/mL
10 ng/mL
1 µM

125 nM
50x
1x
1 µg/mL
2.0 µg/mL
5% (v/v)
1x
10x

Response to the Editor and Reviewers:

We appreciate the careful critiques and inputs provided by Reviewer 1 and the thoughtful comments provided by the Editor. We have addressed their concerns in this revised version of the manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The attached manuscript file has been modified to fit the journal standard.

This version of the manuscript was proofread.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Appropriate changes were made to the Manuscript.

3. Please check and ensure that all the necessary details to reproduce the steps are included in the protocol section (e.g., Line 209/232: How are the cell aggregates transferred?)

Appropriate changes were made to the Manuscript.

4. Please consider splitting the figure into separate Figures. Please use uppercase letters to Label the figure instead of numbers ("1A" instead of "1.1") And label the images within a panel using Roman numbers (I, ii, iii, iv, etc.). Please ensure that scale bare is included in all the panel images and is defined in the figure legends (e.g., In figure 1.3, scale bars are missing).

Figure 1 was split in 2 figures, with an additional panel in figure 2 (see reviewer 2). Labels were changed as suggested and bars were added.

5. Please split the Table of materials and reagents into two separate tables- Table of Materials and Table of Reagents. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Table of materials and reagents was split into two separate tables as suggested. (™) and (®) symbols were removed.

Reviewers' comments

Reviewer #1:

1. In response to culture characterization, the authors expanded on previous experiments from their group and improved clarity in the manuscript itself. However, the response to the poor quality image was to remove the image and add that to the video when it should be to replace the image in the manuscript with a higher quality image.

Higher quality images of hiPSC were included in the first revision of the manuscript. In the current revision, we have included phase contrast images of NPC at an early and late stage of differentiation, as well as the final products of the differentiation protocol (i.e. hiPSC-MN and hiPSC-A).

2. The authors' stated that the MEA recordings would be shown during the video, and while I think a video demonstrating the MEA recordings is informative and helpful for those attempting to understand and use the technique, there's no reason why sample recordings cannot be added to the manuscript as well.

Representative MEA recordings from hiPSC-MN alone and in co-culture with hiPSC-A are now included in a separate figure (figure 3). The analysis of the MEA activity shows that hiPSC-A enhance the electrophysiological maturation of hiPSC-MN.