Application Protocol

Culturing Human iPSC-derived Excitatory Neurons on Microelectrode Arrays: Maestro Pro MEA

Introduction

Elixirgen Scientific's proprietary transcription factor-based technology allows rapid and reproducible differentiation of human iPS or ES cells into neurons without sacrificing the purity of the cells. Our excitatory neurons exhibit typical neuronal morphology with outgrowing neurites and express markers characteristic of a variety of neuronal subtypes, including the pan-neuronal marker tubulin beta 3 class III (TUBB3), the glutamatergic neuron marker vesicular glutamate transporter 1 (vGLUT1), and the cholinergic neuron marker choline acetyltransferase (ChAT).

In this Application Protocol, we describe how our human iPSC-derived excitatory neurons can be thawed, plated, and maintained on the Axion Biosystems Maestro Pro microelectrode array (MEA) system for non-invasive, label-free measurement of neuronal activity. Our neurons fire synchronized bursts within 2 weeks after plating when co-cultured with human primary astrocytes.

Required Equipment and Consumables

Item	Vendor	Catalog Number
Equipment		
Maestro Pro • AxIS Navigator software	Axion Biosystems	
Consumables		
Cytoview MEA 48 Black or White	Axion Biosystems	M768-tMEA-48B or M768-tMEA-48W
4x Quick-Neuron™ Excitatory - Human iPSC-derived Neurons. Each kit contains: • Frozen cells (>1.0 million viable cells, 0.5 ml) • Component N (2x 840 µl) • Component G2 (2x 16 µl) • Component P (14 µl)	Elixirgen Scientific	EX-SeV-CW* (multiple donor cell lines to choose from)
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium**	ThermoFisher	21103049
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140-122
Poly(ethyleneimine) solution, PEI	Sigma-Aldrich	P3143
Pierce™ 20X Borate Buffer	ThermoFisher	28341
Laminin mouse protein, natural	ThermoFisher	23017015
Phosphate-buffered saline (without Ca^{++} Mg^{++})	ThermoFisher	20012050
ROCK inhibitor Y27632	Selleckchem	S1049
B-27™ Plus Neuronal Culture System. This kit contains: • Neurobasal Plus Medium** • B-27™ Plus Supplement (50x)	ThermoFisher	A3653401

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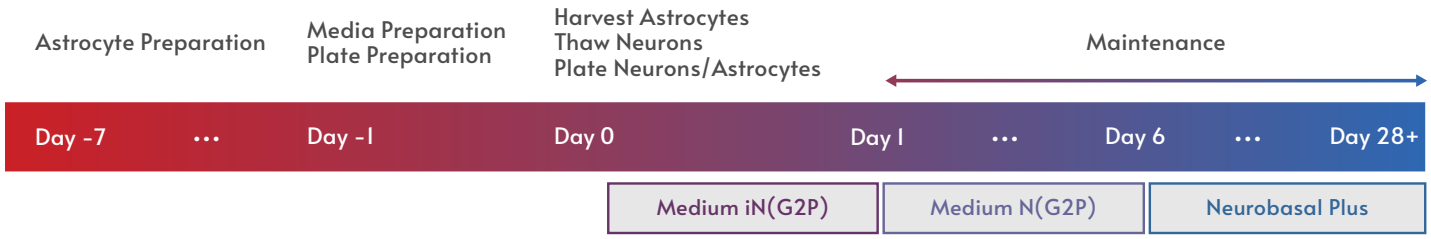
Human Astrocytes Kit. This kit contains: • Human Astrocytes • Astrocyte Medium	ThermoFisher	N7805200
Accutase	ThermoFisher	A1110501
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418
Absolute Ethanol (≥99.5%)	Fisher Scientific	BP2818100
Cell culture-grade water	ThermoFisher	15230162
Ascorbic acid	Sigma-Aldrich	A4544
Syringe filter (0.22 µm)	ThermoFisher	SLGP033RS

* See https://elixirgenscientific.com/ipsc-derived-excitatory-neurons/ for our catalog of iPSC-derived excitatory neurons from healthy and affected donors.

** Please note that Neurobasal and Neurobasal Plus Medium are distinct and are used at different steps of this protocol.

Workflow



Day -7

Astrocyte Preparation

Our neurons fire synchronized bursts within 2 weeks after plating when co-cultured with human primary astrocytes as tested with the Human Astrocytes Kit available through ThermoFisher (Cat No. N7805200). Astrocytes can be prepared by starting their culture one week in advance and harvesting them at Day 0 as instructed by the manufacturer. 1 vial of cryopreserved human primary astrocytes provides a sufficient number of cells (up to 1.5 x 10^6 cells) to plate the Cytoview 48-well plate with our neurons. This protocol assumes that human primary astrocytes are cultured on one 100 mm cell culture dish using manufacturer's recommended protocol.

Day -1

Media Preparation

10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.12 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution is hereafter referred to as iROCK and can be stored at -20°C.

70% EtOH for MEA plate surface treatment

1. Mix Absolute Ethanol (more than 99.5% purity) with cell culture grade water at a ratio of 7 to 3 (e.g., 7 ml Absolute Ethanol with 3 ml cell culture grade water).

1X Borate Buffer

1. Dilute 150 µl of 20X Borate Buffer with 2.85 ml of cell culture-grade water to make 1x Borate Buffer.

0.1% PEI solution

1. Add 5.4 µl PEI to 2.7 ml 1X Borate Buffer.

1 mg/ml laminin stock solution (laminin)

1. Thaw Laminin Mouse Protein, Natural and chill PBS at 4°C or on ice.
2. Mix the Laminin Mouse Protein, Natural and PBS to make the 1 mg/ml stock solution (hereafter referred to as laminin).
    - Laminin concentration varies by lot, so use the number specified on the vial or CoA when making your calculations.
3. Make aliquots of a convenient volume (e.g., 15 µl) and store at -20°C.

200 mM ascorbic acid

1. Dissolve 352 mg ascorbic acid in 10 ml cell culture-grade water.
2. Sterilize using a 0.22 µm syringe filter.
3. Make aliquots of a convenient volume (e.g., 100 µl) and store at -20°C.

Medium N

1. Prepare Medium N using the reagents listed in the table below.
    - Thaw 3 vials of Component N at room temperature for 20-30 minutes or at 4°C overnight.
    - Warm/thaw all other reagents at room temperature for 20-30 minutes.
    - Store Medium N for up to 2 weeks at 4°C. The leftover reagents can be discarded or saved for other uses.

Medium N Reagents	Volume
DMEM/F12	31.2 ml
Neurobasal Medium	31.2 ml
200 mM Glutamax (100x)	325 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	650 µl
Component N	2 ml

Medium N(G2P)

1. Prepare Medium N(G2P) using the reagents listed in the table below.
    - Thaw 3 vials of Component G2 on ice and 2 vials of Component P at room temperature for 20-30 minutes.
    - Medium N(G2P) can be stored for up to 2 weeks when stored at 4°C.
    - Store the rest of Medium N at 4°C for later use. The other leftover reagents can be discarded or saved for other uses.

Medium N(G2P) Reagents	Volume
Medium N	44 ml
Component G2	44 µl
Component P	22 µl

Neurobasal Plus

1. Prepare the maturation medium (hereafter referred to as Neurobasal Plus) using the components listed in the table below.
    - Thaw the B-27™ Plus Supplement (50x) at room temperature for 1 hour or at 4°C overnight.
    - Store Neurobasal Plus for up to 2 weeks at 4°C.
    - **IMPORTANT!** 25 ml of Neurobasal Plus will be sufficient for about 3.5 full plate medium changes. We recommend preparing 25 ml Neurobasal Plus fresh as needed.

Neurobasal Plus Reagents	Volume
Neurobasal Plus Medium	24 ml
B-27™ Plus Supplement (50x)	500 µl
200 mM Glutamax (100x)	250 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	250 µl
200 mM ascorbic acid (filter-sterilized)	25 µl

Plate Preparation

IMPORTANT! For all aspiration steps, use an aspirator with a P200 tip attached to avoid damaging the well bottom center where the electrodes are located.

Surface treatment

1. Add 150 µl 70% EtOH for MEA plate surface treatment prepared above to each well.
    - **IMPORTANT!** Do not use 70% EtOH for common lab use for this step.
2. Incubate the plate at room temperature for 10 minutes.
3. Remove 70% EtOH from all wells and add 150 µl cell-culture grade water to rinse wells.
4. Incubate the plate at room temperature for 10 minutes.
5. Remove the water and leave the plate to dry. Do not turn on UV at the biosafety cabinet.

PEI coating

1. Add 50 µl 0.1% PEI solution to cover electrodes in each well (or per 0.22 cm^2).
2. Incubate the plate at 37°C, 5% CO_2 for 1 hour.
3. Remove 0.1% PEI solution.
4. Add cell-culture grade water to each well and remove it from all wells.
5. Repeat Step 4 for 8 times.
6. Leave the plate inside a biosafety cabinet overnight without closing its lid.

Day 0

Medium iN(G2P)

1. Prepare Medium iN(G2P) by mixing together the following components in a 50 ml conical tube.
    - Warm Medium N(G2P) and 10 mM ROCK inhibitor Y27632 at room temperature for at least 1 hour.

Medium iN(G2P) Reagents	Volume
Medium N(G2P)	20 ml
iROCK	20 µl

Harvesting Human Primary Astrocytes

1. Remove culture medium from the dish with human primary astrocytes and add 10 ml PBS.
2. Remove PBS and add 3 ml Accutase to the dish.
3. Incubate the dish at 37°C, 5% CO_2 for 5 minutes.
4. Add 3 ml astrocyte culture medium to the dish.
5. Disperse the medium quickly over the bottom surface of the well by pipetting 6-8 times to detach cells using a P1000 pipettor.
6. Collect 6 ml cell suspension in a 15 ml conical tube using the same P1000 pipettor.
7. Count the cells and determine quantity and viability. While counting the cells, the cell suspension should be kept on ice.
8. Centrifuge the conical tube at 200 xg for 4 minutes.
9. Aspirate most of the supernatant from the conical tube but leave a small volume (<50 µl) to cover the pellet.
10. Gently tap the side of the conical tube to break the cell pellet.
11. Prepare 264 µl of a 4.0 x 10^6 live cells/ml cell suspension using Medium iN(G2P). If the total volume exceeds 264 µl or cannot reach the cell density to 4.0 x 10^6 live cells/ml, centrifuge the excess volume at 200 xg for 4 minutes and resuspend the pellet to adjust the volume to 4.0 x 10^6 live cells/ml.
12. Gently tap the side of the conical tube and pipet up and down 2-3 times.
13. Keep the astrocytes on ice until ready for plating.

Thawing neurons

1. Warm Medium N at room temperature for 20-30 minutes.
2. Take out the 4 cryovials of frozen human iPSC-derived excitatory neurons from the liquid nitrogen storage tank.
3. Incubate the cryovials in a water bath set at 37°C (do not submerge the cap) until most of the contents are thawed but a small ice crystal remains (1.5~2 minutes).
4. Wipe the vials with a dry paper towel. Spray 70% ethanol on the vials and bring them inside a biosafety cabinet.
5. Using a P1000 pipettor, gently transfer the cell suspension from each vial to a 50 ml conical tube.
6. Take 1 ml Medium N and add it to each original cryovial. Pipet up and down 2-3 times and then transfer the medium to the 50 ml conical tube with the cell suspension dropwise at 1 drop per 1-2 seconds.
7. Take 16 ml Medium N (4 ml per cryovial) and add it into the 50 ml conical tube dropwise at 1 drop/1-2 seconds while gently shaking the tube.
8. Gently pipet the cell suspension up and down 2-3 times using a 5ml pipettor.
9. Count the cells and determine quantity and viability. While counting the cells, the cell suspension should be kept on ice.
10. Centrifuge the conical tube at 200 xg for 4 minutes.
11. Aspirate most of the supernatant from the conical tube but leave a small volume (<50 µl) to cover the pellet.
12. Gently tap the side of the conical tube to break the cell pellet.
13. Prepare 264 µl of a 1.6 x 10^7 live cells/ml cell suspension using Medium iN(G2P). If the total volume exceeds 264 µl or cannot reach the cell density to 1.6 x 10^7 live cells/ml, centrifuge the excess volume at 200 xg for 4 minutes and resuspend the pellet to adjust the volume to 1.6 x 10^7 live cells/ml.
14. Gently tap the side of the conical tube and pipet up and down 2-3 times.

Plating Neurons with Human Primary Astrocytes

1. Add 264 µl human primary astrocyte cell suspension to the human iPSC-derived neuron cell suspension.
2. Add 10.6 µl laminin into the cell suspension on ice to make a final laminin concentration 20 µg/ml. Mix well.
    - **IMPORTANT!** Cells start aggregating immediately after adding laminin. Plate cell suspensions in all wells within 10 minutes to prevent cells clumping together.
3. Plate 10 µl cell suspension to cover the electrodes in each well. Incubate the cultures at 37°C, 5% CO_2 for at least 1 hour or up to 3 hours until cells settle down.
4. Add 300 µl Medium iN(G2P) to each well.
5. Add 5 ml PBS to the edge of the plate to keep from drying out.
6. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 1

Maintenance

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well using a P1000 pipettor and add 500 µl PBS to wash away any cellular debris.
3. Pipet out PBS from each well using a P1000 pipettor and add 300 µl Medium N(G2P).
4. Incubate the cultures at 37°C, 5% CO_2 for 3 days.

Day 4

Maintenance

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out 50% old medium (150 µl) from each well using a P1000 pipettor and add 150 µl Medium N(G2P).
3. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 6+

Maintenance

1. Warm Neurobasal Plus at room temperature for 20-30 minutes.
2. Pipet out 50% old medium from each well using a P1000 pipettor and add 150 µl Neurobasal Plus to it.
3. Incubate the cultures at 37°C, 5% CO_2 for 3-4 days.
4. Repeat Step 1 to 4 twice a week such as on Tuesday and Friday.