Application Protocol

Culturing human iPSC-derived mixed neurons on multielectrode arrays: MED64 Presto MEA

Introduction

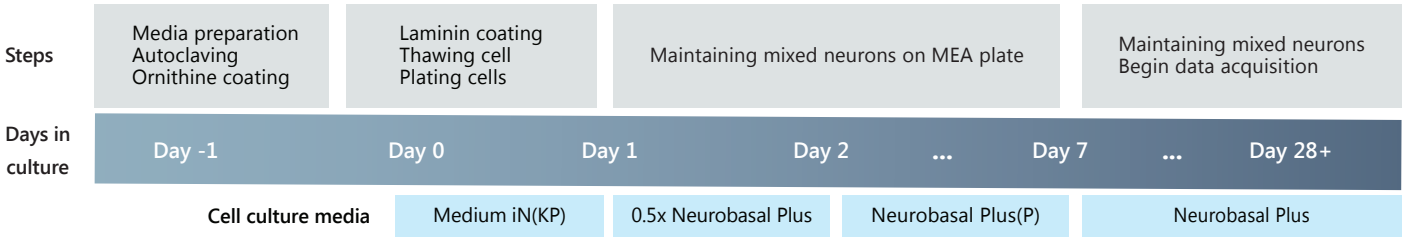
Elixirgen Scientific's proprietary transcription factor-based technology allows rapid and reproducible differentiation of human iPSCs into neurons without sacrificing the purity of the cells. Our Mixed Neurons exhibit typical neuronal morphology with outgrowing neurites and express markers characteristic of a variety of neuronal subtypes, including the pan-neuronal marker tubulin beta 3 class III (TUBB3), the cholinergic neuron marker choline acetyltransferase (ChAT), the dopaminergic neuron marker tyrosine hydroxylase (TH), the GABAergic neuron marker glutamate decarboxylase 1 (GAD67/GAD1), the glutamatergic neuron marker vesicular glutamate transporter 1 (vGLUT1), and the serotonergic neuron marker tryptophan hydroxylase 2 (TPH2). In this Application Protocol, we describe how our human iPSC-derived Mixed Neurons can be thawed, plated, and maintained on the AlphaMED Scientific MED64 Presto multielectrode array (MEA) system for non-invasive, label-free measurement of neuronal activity.

Required equipment and consumables

Item	Vendor	Catalog number
MED64 Presto System - MEA Symphony software	AlphaMED Scientific	
24-well MED plate for MED64 Presto-eco	AlphaMED Scientific	MED-Q2430M
CellSpotter	AlphaMED Scientific	MED-CRS24M
Cloning Ring Id 3.4mm, RING-05	AGC Techno Glass (IWAKI)	61-9714-50
Mixed Neurons from Human iPSCs.* The kit contains: - Frozen cells (>1.0 million viable cells, 0.5 ml) - Component N (840 µl) - Component K (14 µl) - Component P (25 µl)	Elixirgen Scientific	EXGS-QNMSVF-CW_ID_-1M (multiple cell lines to choose from)
DMEM/F12	ThermoFisher	21331-020
Neurobasal**	ThermoFisher	21103049
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140-122
Poly-L-Ornithine	Sigma-Aldrich	P4957-50ML
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
Trypan blue solution, 0.4%	ThermoFisher	15250061
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418
Cell culture-grade water	ThermoFisher	15230162
Ascorbic acid	Sigma-Aldrich	A4544
Syringe filter (0.22 µm)	ThermoFisher	SLGP033RS

*See elixirgenscientific.com/store/category/mixed-neurons/ for a full list of mixed neuron products derived from human iPSCs.
**Please note that Neurobasal and Neurobasal Plus Medium are distinct and are used at different steps of this protocol.

Workflow



Methods

Media preparation

Component N and B-27™ Plus Supplement (50x) should be thawed overnight at 4°C before being used to prepare Medium N and Neurobasal Plus, respectively. To keep with the above workflow, we recommend thawing Component N and the B-27™ Plus Supplement (50x) overnight 1-2 days before plating.

Preparing the plating medium (Medium N)

1. Thaw Component N at 4°C overnight.
2. Prepare the plating medium (hereafter referred to as Medium N) using the components listed in the table below.
3. Store Medium N for up to 2 weeks when stored at 4°C.

Medium N components	Volume
DMEM/F12	12 ml
Neurobasal	12 ml
200 mM Glutamax (100x)	125 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	250 µl
Component N	775 µl

Preparing 10 mM ROCK inhibitor Y27632

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.1225 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl) and store at -20°C.

Preparing 0.002% poly-L-ornithine solution (ornithine)

1. Take 1 ml 0.01% poly-L-ornithine solution and mix it with 4 ml PBS.
2. Store the 0.002% poly-L-ornithine solution (hereafter referred to as ornithine) for up to 2 weeks at 4°C.

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

Preparing 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.

Preparing the maturation medium (Neurobasal Plus)

1. Thaw the B-27™ Plus Supplement (50x) at 4°C overnight.
2. Prepare the maturation medium (hereafter referred to as Neurobasal Plus) using the components listed in the table below.
3. Store Neurobasal Plus for up to 2 weeks at 4°C.

Neurobasal Plus Components	Volume
Neurobasal Plus Medium	48 ml
B-27™ Plus Supplement (50x)	1 ml
200 mM Glutamax (100x)	0.5 ml
Penicillin-Streptomycin (10000 units/ml; 100x)	0.5 ml
200 mM ascorbic acid (filter-sterilized)	50 µl

Day -1

Autoclaving

1. Wrap 25 cloning rings with aluminum foil and autoclave.
2. Wrap CellSpotter with aluminum foil and autoclave.

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

Ornithine coating

1. Add ~0.9 ml 70% EtOH into each well and leave the plate inside the biosafety cabinet for 10 min.
2. Tilt the plate and aspirate EtOH completely off at the side of the well.
3. Sterilize MEA plate and its lid (inside facing up) under UV light for 15-30 min.
4. Rinse each well 4 times with ~300 µl MilliQ water (~8 ml for 24 wells).
5. Add 200 µl ornithine to each well (or per 0.95 cm^2).
6. Incubate the plate at 37°C, 5% CO_2 overnight (or at least 2 hours if coating on Day 0).

Day 0

Laminin coating

1. Thaw Laminin and chill PBS on ice for 20-30 minutes.
2. Combine 5 ml ice-cold PBS with 50 µl Laminin and mix well.
3. Aspirate the supernatant from each well and add 300 µl PBS to each of the 24 wells.
4. Repeat step 3.
5. Aspirate PBS from each well and add 200 µl diluted Laminin to it (or per 0.95 cm^2).
6. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours.
7. When cells are ready for plating, repeat step 3 twice, and then proceed to the “Plating mixed neurons” section.

Preparing Medium iN(KP)

1. Prepare Medium iN(KP) by mixing together the following components in a 15 mL conical tube.
    a. Warm Medium N at room temperature for at least 1 hour and thaw 10 mM ROCK inhibitor Y27632 and Components K and P at room temperature for 20-30 min before combining.
    b. Store leftover Components K and P at 4°C for later use.

Medium iN(KP) Components	Volume
Medium N	4 ml
10 mM ROCK inhibitor Y27632	4 µl
Component K	4 µl
Component P	2 µl

Thawing mixed neurons

1. Take out the vial of Frozen Cells from the liquid nitrogen storage tank.
2. Incubate the cryovial in a water bath set at 37°C (do not submerge the cap) until most of the content is thawed but a small ice crystal remains (~2 minutes).
3. Wipe the vial with dry paper towel. Spray 70% ethanol to the vial and bring it inside a biosafety cabinet.
4. Take 4.5 ml Medium N into a new 15 ml conical tube and store the rest of Medium N at 4°C for later use.
5. Using a P1000 pipettor, take 0.5 ml Medium N and add it into the cryovial dropwise at 1 drop/1-2 seconds.
    a. Use the same pipette tip for Steps 5-8 to minimize cell loss.
6. Gently pipet the cell suspension up and down once before transferring to the conical tube containing Medium N.
7. Take 1 ml cell suspension from the conical tube, add it to the original cryovial, and pipet up and down 2-3 times before transferring the entire volume back to the conical tube.
8. Mix the cell suspension by pipetting up and down 3 times.
9. Centrifuge the conical tube at 200 xg for 4 minutes.
10. Aspirate most of the supernatant from the conical tube but leave a small volume (<50 µl) to cover the pellet.
11. Gently tap the side of the conical tube to break the cell pellet.
12. Add 1 ml Medium iN(KP) to the conical tube using a P1000 pipettor and pipet up and down 2-3 times.
13. Count cells and determine cell viability by trypan blue staining.

Plating mixed neurons

> *It is very important to plate cells one well at a time to prevent air drying of laminin, which will result in neuron aggregation.*

1. Determine the volume of cell suspension for 6 x 10^4 viable cells for each well to start a culture(s). If the volume exceeds 20 µl per well, centrifuge the excess volume at 200 xg for 4 minutes and resuspend the pellet to adjust the volume to 20 µl per well (each cloning ring can hold up to 90 µl).
2. Place an autoclaved cloning ring (Φ3.4mm) to each well through CellSpotter to plate cells around electrodes.
    a. Each slit on the well should be facing you.
3. Aspirate most, but not all of, the PBS (<200 µl) from one coated well and place an autoclaved cloning ring (Φ3.4mm) to the well through CellSpotter to plate cells around electrodes.
4. Take out the determined volume of the cell suspension from Step 1 and plate it inside each cloning ring.
    a Use a P10 tip to prevent air bubbles from being trapped inside the cloning ring.
5. Working one well at a time, repeat Steps 3 and 4 for the rest of the wells.
6. Add 200 µl Medium iN(KP) into each well outside the cloning ring through the slit of CellSpotter.
7. Incubate the cultures at 37°C, 5% CO_2 for 1-3 hours or until cells settle down.
8. Remove the cloning ring from each well and incubate the cultures at 37°C, 5% CO_2 overnight.

Maintaining mixed neurons on MEA Plate (Days 1-7+)

Day 1

1. Warm Medium N, Component K, Component P, and Neurobasal Plus at room temperature for 20-30 minutes.
2. Prepare 0.5x Neurobasal Plus using the components listed in the table below.

0.5x Neurobasal Plus Components	Volume
Medium N	2.5 ml
Neurobasal Plus	2.5 ml
Component K	2.5 µl
Component P	2.5 µl

3. Pipet out the old medium from each well using a P1000 pipettor and add 500 µl PBS to it to wash away any cellular debris.
4. Pipet out PBS from each well using a P1000 pipettor and add 200 µl 0.5x Neurobasal Plus to it.
5. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 2

1. Warm Neurobasal Plus and Component P at room temperature for 20-30 minutes.
2. Mix 15 ml Neurobasal Plus and 7.5 µl Component P together. The medium is hereafter referred to as Neurobasal Plus(P).
3. Pipet out the old medium from each well using a P1000 pipettor and add 400 µl Neurobasal Plus(P) to it.
4. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 4

1. Warm Neurobasal Plus(P) at room temperature for 20-30 minutes.
2. Pipet out 50% old medium from each well using a P1000 pipettor and add 200 µl Neurobasal Plus(P) to it.
3. Incubate the cultures at 37°C, 5% CO_2 for 2-3 days.

Day 7+

1. Warm Neurobasal Plus (without Component P) at room temperature for 20-30 minutes.
2. Pipet out old medium from each well using a P1000 pipettor and add 400 µl Neurobasal Plus to it.
3. Incubate the cultures at 37°C, 5% CO_2 for 2-3 days.
4. Repeat steps 1-3 every 2-3 days.

Data acquisition

Neuronal activity can be acquired and analyzed with the MEA Symphony software according to the manufacturer's guidelines. Spontaneous action potentials can be observed in some wells as early as day 10 post-plating. Synchronized bursts can be observed in some wells by day 14 and strong network bursts can be observed by day 28. Reproducible drug responses can be measured between days 42-56.

Technical support

For technical support, please contact us at cs@elixirgenscientific.com or call us at +1 (443) 869-5420 (M-F 9am-5 pm EST).

Last revised: December 2019