Application Protocol

Culturing Human iPSC-derived Mixed Neurons on Multielectrode Arrays: MaxTwo Multiwell 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, 67 kDa isoform (GAD1/GAD67), the glutamatergic neuron marker vesicular glutamate transporter 1 (vGLUT1), and the serotonergic neuron marker tryptophan hydroxylase 2 (TPH2).

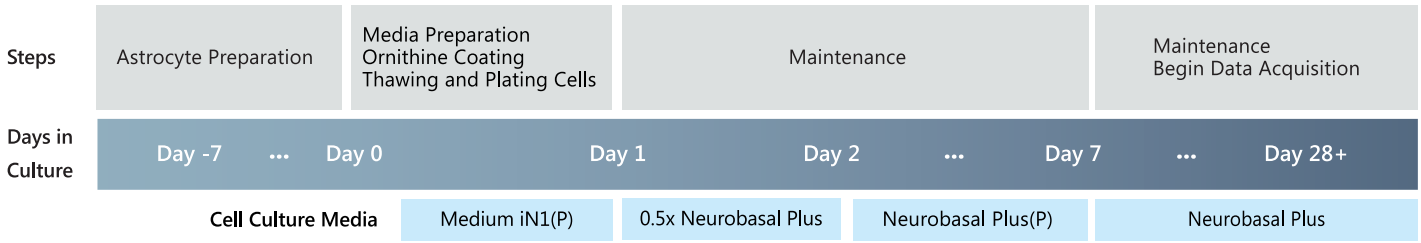
In this Application Protocol, we describe how our human iPSC-derived Mixed Neurons can be thawed, plated, and maintained on the MaxWell Biosystems MaxTwo Multiwell multielectrode array (MEA) system for non-invasive, label-free measurement of neuronal activity. Our Mixed Neurons fire synchronized bursts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

Required Equipment and Consumables

Item	Vendor	Catalog Number
MaxTwo Multiwell MEA System - MaxLab Live Basic software, perpetual license	MaxWell Biosystems	MX2-SYS
MaxTwo 6-well Plates	MaxWell Biosystems	MX2-S-6W
Mixed Neurons from human iPSCs.* The kit contains: - Frozen cells (>1.0 million viable cells, 0.5 ml) - Component N1 (830 µl) - Component P (14 µl)	Elixirgen Scientific	MN-SeV-C# (multiple donor cell lines to choose from) Formerly EXGS-QNMSVF-CW#
DMEM/F12	ThermoFisher	21331-020
Neurobasal**	ThermoFisher	21103049
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140-122
Poly-L-Ornithine Solution, 0.01%	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
Human Astrocytes Kit. This kit contains: - Human Astrocytes - Astrocyte Medium	ThermoFisher	N7805200
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 https://elixirgenscientific.com/ipsc-derived-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



IMPORTANT! Component N1 and B-27™ Plus Supplement (50x) should be thawed overnight at 4°C before being used to prepare Medium N1 and Neurobasal Plus, respectively. To keep with the above workflow, we recommend thawing Component N1 and the B-27™ Plus Supplement (50x) overnight 1-2 days before plating.

Astrocyte Preparation

Our Mixed Neurons fire synchronized bursts within 2 weeks after plating when co-cultured with human primary astrocytes as tested with 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. Primary astrocytes have a limited capability to proliferate (i.e., mature astrocytes). However, 1 vial of cryopreserved human primary astrocytes provides a sufficient number of cells (up to 1.5 x 10^6 cells) to plate the MaxTwo 6-well plate with our Mixed Neurons.

Media Preparation

Preparing the plating medium (Medium N1)

1. Thaw Component N1 at 4°C overnight.
2. Prepare the plating medium (hereafter referred to as Medium N1) using the components listed in the table below.
3. Store Medium N1 for up to 2 weeks when stored at 4°C.

Medium N1 Components	Volume
DMEM/F12	12 ml
Neurobasal	12 ml
N2 Supplement (100x)	125 µl
200 mM Glutamax (100x)	62.5 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	250 µl
Component N1	750 µl

Preparing 10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.1225 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.

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

1. Take 300 µl 0.01% poly-L-ornithine solution and mix it with 1.2 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 at 4°C or on ice and chill PBS at 4°C or on ice.
2. Take cold Laminin Mouse Protein, Natural and mix it with chilled PBS to make a 1 mg/ml stock solution hereafter referred to as laminin.
    - **Note**: The concentration of laminin varies among different lots and is specified on the vial or its certificate of analysis. Calculate the volume needed to prepare 1 mg/ml stock solution accordingly.
3. Make aliquots of a convenient volume (e.g., 15 µl) and store them 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 0

Sterilization of the MaxTwo plate

1. Spray the MaxTwo plate and the lid with 70% EtOH and then put them inside the biosafety cabinet.
2. Fill all wells, areas outside the wells on the plate, and the lid with 70% EtOH all the way up to the edges and leave them for 30 minutes inside the biosafety cabinet.
    - About 6-7 ml 70% EtOH per well (~50 ml total) is needed.
3. Remove 70% EtOH from the plate and the lid by decanting into a liquid waste beaker.
4. Rinse the wells with cell-culture grade water 3 times.
5. Air dry the plate and the lid under the laminar air flow inside the biosafety cabinet without the UV light turned on for 30 minutes.

Ornithine and laminin coating

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. Do not scratch the electrodes.

1. Wet the electrodes at the center of each well with 10 µl cell-culture grade water by quickly pipetting it in and out three times.
2. Add 30 µl ornithine to cover electrodes at the center of each well (or per 2x4 mm^2).
3. Add 5 ml cell culture-grade water to the area outside the wells on the plate to keep humidity inside the wells.
4. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours.
5. Mix 6 µl Laminin with 69 µl Medium iN1(P) prepared as described below.
6. Add 300 µl cell culture-grade water to each of the ornithine-treated wells.
7. Aspirate water from each well and add cell culture-grade water as described at the previous step.
8. Aspirate water from each well completely and leave the plate in the biosafety cabinet for 30 minutes to allow air drying of the wells.
9. Add 10 µl Laminin diluted in Medium iN1(P) as prepared at Step 6 to cover electrodes at the center of each well.
10. Incubate the plate at 37°C, 5% CO_2 for at least 30 minutes or until cells become ready for plating.

Preparing Medium iN1(P)

1. Prepare Medium iN1(P) by mixing together the following components in a 15 mL conical tube.
    - Warm Medium N1 and iROCK at room temperature for at least 1 hour and thaw Component P at room temperature for 20-30 min before combining.
    - Store leftover Components P at 4°C for later use.

Medium iN1(P) Components	Volume
Medium N1	4.5 ml
iROCK	4.5 µl
Component P	2.25 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 N1 into a new 15 ml conical tube and store the rest of Medium N1 at 4°C for later use.
5. Using a P1000 pipettor, take 0.5 ml Medium N1 and add it into the cryovial dropwise at 1 drop/1-2 seconds.
    - 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 N1.
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 iN1(P) 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 with human primary astrocytes

1. Determine the total volume of cell suspensions for 2 x 10^5 viable Mixed Neurons and 0.5 x 10^5 viable human primary astrocytes for each well. If the total volume exceeds 10 µl per well, mix the two cell suspensions, centrifuge them at 200 xg for 4 minutes, and re-suspend the pellet to adjust the volume to 10 µl per well.
2. Plate 10 µl cell suspension onto the drop of Laminin to cover electrodes in each well.
3. 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 500 µl iN(P) to each well and incubate the cultures at 37°C, 5% CO_2 overnight.

Day 1

Maintenance

1. Warm Medium N1, 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 N1	3 ml
Neurobasal Plus	3 ml
Component P	3 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 500 µl 0.5x Neurobasal Plus.
5. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 2

Maintenance

1. Warm Neurobasal Plus and Component P at room temperature for 20-30 minutes.
2. Mix 10 ml Neurobasal Plus and 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 1 ml Neurobasal Plus(P).
4. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 4

Maintenance

1. Warm Neurobasal Plus(P) at room temperature for 20-30 minutes.
2. Pipet out 50% old medium (i.e., 500 µl) from each well using a P1000 pipettor and add 500 µl Neurobasal Plus(P).
3. Incubate the cultures at 37°C, 5% CO_2 for 2-3 days.

Day 7+

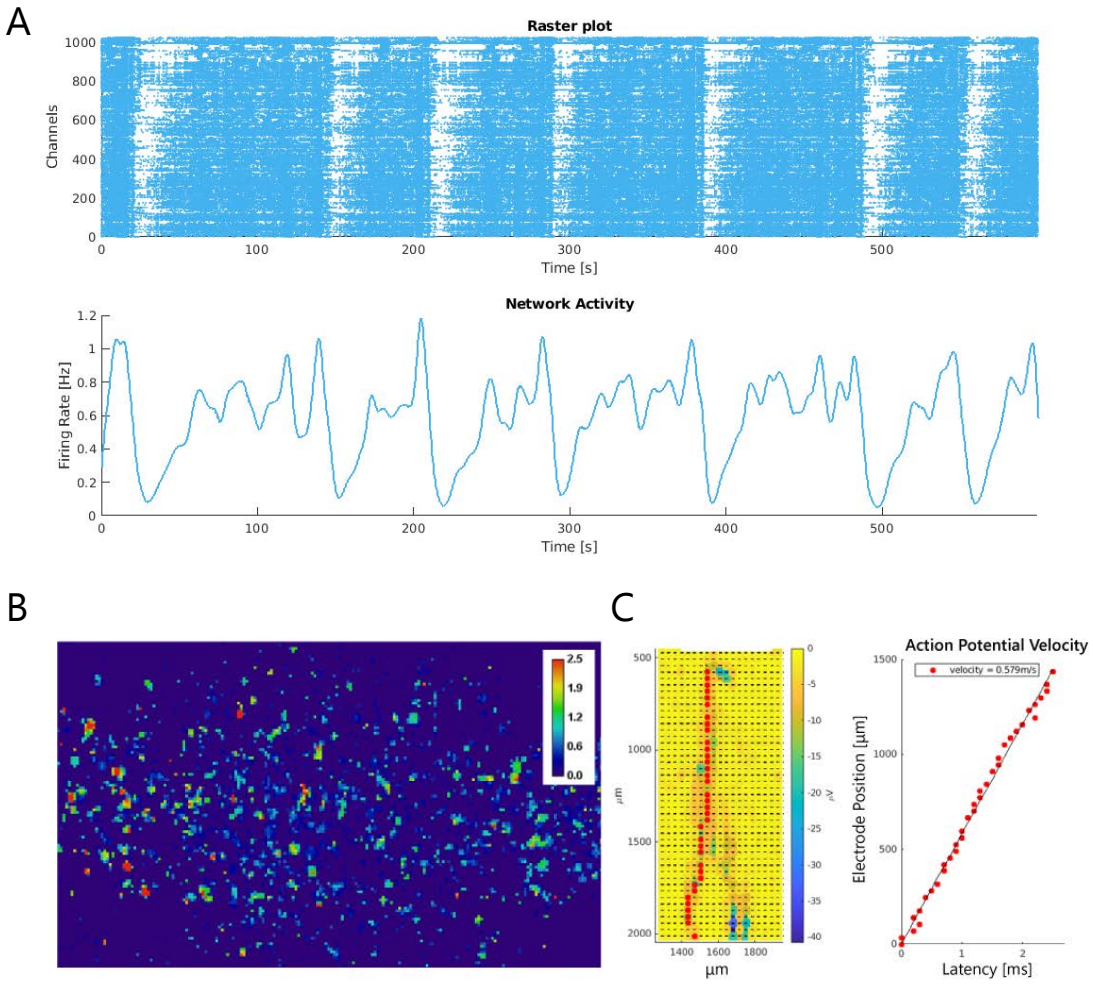
Maintenance

1. Warm Neurobasal Plus (without Component P) at room temperature for 20-30 minutes.
2. Pipet out most of the old medium from each well using a P1000 pipettor and add 1 ml Neurobasal Plus.
3. Incubate the cultures at 37°C, 5% CO_2 for 2-3 days.
4. Replace 50% of the old medium with fresh Neurobasal Plus every 3-4 days.

Data Acquisition

Neuronal activity can be acquired and analyzed with the MaxLab Live Basic software according to the manufacturer's guidelines. Spontaneous action potentials can be observed in some wells as early as Day 10 and synchronized network bursts by Day 14.

Representative Data



Electrophysiological activities of hiPSC-derived mixed neurons. Mixed neurons (2×10^5 cells) were co-cultured with astrocytes (5×10^4) as described in this Application Protocol and their activity was measured using the MaxTwo Multiwell HD-MEA (MaxWell Biosystems). (A) After scanning spontaneous firing of axon potential for the entire electrodes, about 1,000 electrodes were selected for scanning their network burst activity. The raster plot indicates firing of strong synchronized bursts over the duration of 10 minutes on Day 43. (B) The corresponding result of activity scan shows that active neurons are evenly distributed over the entire chip on Day 43. (C) By tracing the propagation of axon potential in a single neuron, action potential velocity was analyzed. This graph indicates that the action potential propagated rapidly along the long axon (~1.5 mm), demonstrating the maturity of neurons and their suitability for axonal conduction studies.

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: May 13, 2020