Co-Culture of Human Neurons and Astrocytes

MATERIALS PROVIDED BY BRAINXELL

- One vial of 5 million cryopreserved human neurons (200 µL)
- One vial of 2 million cryopreserved human astrocytes (200 µL)
- 100 µL Neuron Seeding Supplement at 1000X
- 50 µL Neuron Day 4 Supplement at 1000X
- 150 µL Astrocyte Supplement at 1000X

ADDITIONAL MATERIALS NEEDED

- DMEM/F12 Medium (Life Technologies #11330-032)
- Neurobasal Medium (Life Technologies #21103-049)
- B27 Supplement (Life Technologies #17504-044)
- N2 Supplement (Life Technologies #17502-048)
- GlutaMAX (Life Technologies #35050-061)
- Geltrex (Life Technologies #A1413201)
- BDNF (Peprotech #450-02)
- GDNF (Peprotech #450-10)
- TGF-β1 (Peprotech #100-21C)
- PDL-Coated 96-Well Plates

RECOMMENDED PROCEDURE

Thawing and Seeding the Neurons

1. Gather the components for the Seeding Medium according to the recipe below. Note that BDNF, GDNF, and TGF-β1 are supplied as lyophilized powders. Follow the manufacturer's instructions for reconstitution. We recommend creating stock solutions of 10 µg/mL for BDNF, 10 µg/mL for GDNF, and 1 µg/mL for TGF-β1.
2. Thawing neurons and astrocytes and seeding of one 96-well plate requires approximately 20 mL of seeding medium. Working in a cell culture hood (biological safety cabinet), combine all components in a sterile 50 mL conical tube. Allow the medium to equilibrate to room temperature for 15 minutes. Do not warm the medium in a 37°C water bath.
3. Remove the cryovials from the liquid nitrogen and place in a 37°C water bath. To minimize contamination, avoid submerging the caps. Gently move the vials within the bath to increase the rate of thawing.
4. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the vials from the water bath. Disinfect the vials by spraying them with 70% ethanol and transfer it to the cell culture hood.
5. Slowly add 800 µL of seeding medium to each vial at a rate of ~1 drop/s using a 1 mL pipette tip. This process should take about 30 seconds per vial.
6. Gently transfer all contents (1mL total) from each vial to new sterile 50 mL conical tubes.
7. To collect any residual cells, gently add another 1 mL of seeding medium to each vial and then transfer to the coinciding conical tube.

8. Slowly add an additional 3 mL of seeding medium to each 50 mL conical tube using a 10 mL serological pipette. Gently swirl the conical tube while adding the medium. This process should take about 1 minute.
9. To count the cells, gently swirl the conical tubes again and remove 10 µL from the cell suspension. Count the number of viable cells per mL with a hemocytometer using the trypan blue exclusion method to identify dead/viable cells.
10. The recommended seeding density is 10,000-20,000 viable neurons/well with 5,000 viable astrocytes/well for a 96-well plate (~30,000-60,000 viable neurons/cm^2 and ~15,000 viable astrocytes/cm^2). Calculate the volume of each cell suspension needed to prepare a 12 ml mixture, which will be enough to seed one 96-well plate.
11. In a new 50 mL conical tube, add the calculated volume of each cell suspension needed for neurons and astrocytes, and then add enough medium to obtain a final volume of 12 mL. For example, if the volume of the neuron cell suspension needed is 2 mL and the volume of the astrocyte cell suspension is 0.5 ml, combine 2 mL of neurons, 0.5 ml of astrocytes, and 9.5 mL of fresh seeding medium.
12. Mix completely and then plate 100 µL/well (20,000 cells/well) onto a PDL-coated 96-well plate using a multi-channel pipettor or liquid handler. Throughout the seeding process, be careful not to move or agitate the plate as this may lead to uneven attachment.
13. After seeding, do not immediately transfer the plate to the incubator. Leave it in the hood for 15 minutes to allow the cells to settle to the bottom of the well. After 15 minutes, very gently transfer the plate to a humidified incubator at 37°C with 5% CO_2.
14. Note: the day the cells are plated is designated as Day 0.

Day 1 Medium Change

1. On Day 1 (24 hours after seeding), prepare fresh medium according to the Day 1 Medium composition below. Note that this medium is the same as the Seeding Medium except for the addition of Geltrex.
2. To prepare the Geltrex, add cold DMEM/F12 directly to an aliquot of frozen Geltrex to yield a 1:10 dilution. For example, if aliquots of Geltrex have a volume of 100 µL, add 900 µL of cold DMEM/F12. Immediately place this mixture at 4°C to allow the Geltrex to thaw and dissolve.
3. Very gently remove the entire 100 µL from each well and gently add 100 µL of the Day 1 Medium back to each well. Take care to ensure that the neurons do not dry out at any point in the medium changing process. We recommend changing one row or column at a time using a multichannel pipettor. Once complete, return the plate to the incubator.

Day 4 Medium Addition

1. On Day 4 (96 hours after seeding), prepare fresh medium following the Day 4 recipe described below, which includes astrocyte supplement and Day 4 supplement, but does not require seeding supplement or Geltrex.
2. Gently add 100 µL/well to the entire plate for a total of 200 µL/well.

Day 7 and Onward Medium Changes

1. Change half the medium (100 µL/well) twice weekly (on Day 7, 11, 14, 18, etc.). The medium for these changes is the same as above excluding the supplements and Geltrex (see recipe below).
2. The neurons mature rapidly can be maintained viable and adherent in culture under the above conditions for at least 4 weeks post-seeding.

Media Compositions

		Component	Stock Conc.	Final Conc.	Volume
Seeding Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL
	3	B27 Supplement	50X	1X	400 µL
	4	N2 Supplement	100X	1X	200 µL
	5	GlutaMAX	200 mM	0.5 mM	50 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL
	9	Seeding Supplement	1000X	1X	20 µL
	10	Astrocyte Supplement	1000X	1X	20 µL

		Component	Stock Conc.	Final Conc.	Volume
Day 1 Medium	1	DMEM/F12 Medium	1X	0.5X	9.5 mL
	2	Neurobasal Medium	1X	0.5X	9.5 mL
	3	B27 Supplement	50X	1X	400 µL
	4	N2 Supplement	100X	1X	200 µL
	5	GlutaMAX	200 mM	0.5 mM	50 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL
	9	Seeding Supplement	1000X	1X	20 µL
	10	Astrocyte Supplement	1000X	1X	20 µL
	11	Geltrex	15 mg/mL	15 µg /mL	200 µL (of 1:10)

		Component	Stock Conc.	Final Conc.	Volume
Day 4 Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL
	3	B27 Supplement	50X	1X	400 µL
	4	N2 Supplement	100X	1X	200 µL
	5	GlutaMAX	200 mM	0.5 mM	50 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL
	9	Astrocyte Supplement	1000X	1X	20 µL
	10	Day 4 Supplement	1000X	1X	20 µL

		Component	Stock Conc.	Final Conc.	Volume
Day 7 and Onward Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL
	3	B27 Supplement	50X	1X	400 µL
	4	N2 Supplement	100X	1X	200 µL
	5	GlutaMAX	200 mM	0.5 mM	50 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL

ALTERNATIVE APPROACH

Under some circumstances it may be beneficial to pre-plate astrocytes and then add neurons to the same plate several days later. This is the recommended approach if performing an imaging assay and planning to culture the neurons for less than 14 days. When pre-plating astrocytes, BrainXell's Astrocyte Culture Protocol can be followed directly for thawing, seeding, and Day 1 medium change. On Day 4 we recommend adding the neurons to the plate. When adding neurons on Day 4 after astrocyte pre-plating, follow the thawing and seeding instructions described in BrainXell's Neuron Cell Culture Protocols. The astrocyte supplement should be added to the seeding medium when plating the neuron. From this point forward, simply follow the neuron culture protocol in place of the astrocyte protocol.