96-well Spinal Neuron/Astrocyte Co-culture Assay

CONTENTS

- One vial of 5 million cryopreserved human neurons (500 µL)
- One vial of 2 million cryopreserved human astrocytes (500 µL)
- Neuron Seeding Supplement at 1000X
- Neuron Day 4 Supplement at 1000X
- Astrocyte Supplement at 1000X
- Supplement K at 1000X

Immediately transfer the vial of neurons to liquid nitrogen upon receipt. Transfer the vials of supplements to a -20°C freezer. The supplements can be stored at -20°C for up to 6 months. Alternatively, the supplements can be stored at -80°C for up to 18 months.

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

If performing spheroid cultures, the recommended plates are either S-Bio PrimeSurface 3D Culture Spheroid (ULA) plates (CAT: MS-9096WZ) or Nunclon Sphera 96-Well, U-Shaped-Bottom Microplate (CAT: 174925). When seeding these plates cells can be seeded between 100 and 10,000 cells/well successfully.

Thawing and Seeding the Neurons and Astrocytes

1. Gather the components for the Seeding Medium according to the recipe below. Note that BDNF, GDNF, and TGF-β1 are 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. Working in a cell culture hood (biological safety cabinet), combine all components in an appropriately sized sterile container. For preparation of 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 before adding the appropriate amount to the Seeding Medium. Allow the Seeding Medium to equilibrate to room temperature for at least 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.
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 500 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. For each respective cell type, perform a cell count. Gently swirl the conical tube 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. Repeat count for other cell type.
10. Combine human neurons and human astrocytes at your desired ratio. We recommend a 4:1 ratio, but other ratios may be suitable depending on user preference.
11. The recommended seeding density is 25,000 – 40,000 (i.e. 20,000 – 32,000 human neurons with 5,000 – 8,000 human astrocytes) viable cells/well for a 96-well plate (~80,000 – 125,000 viable cells/cm^2). Use the following equation to determine the volume of cell suspension needed for each 96-well plate: volume of cell suspension needed (mL) = (3.0 – 4.8x10^6 cells)/(viable cells per mL).
12. 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.
13. Mix completely and then plate 100 µL/well (25,000 – 40,000 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.
14. 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. Day of cell plating is designated as Day 0.

    *Note: Entire thawing and plating process should not exceed 2 hours, post-thaw viability and overall cell health will be severely impacted and lead to an unsuccessful culture.

Day 4 Medium Addition

1. On Day 4 (96 hours after seeding), prepare fresh Day 4 Medium (see recipe below).
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.) using maintenance medium (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.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Seeding Medium	1	DMEM/F12 Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	2	Neurobasal Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL	40 µL	100 µL
	9	Geltrex	15mg/mL	15 µg/mL	200 µL (of 1:10)	400 µL (of 1:10)	1 mL (of 1:10)
	10	Neuron Seeding Supplement	1000X	1X	20 µL	40 µL	100 µL
	11	Astrocyte Supplement	1000X	1X	20 µL	40 µL	100 µL
	12	Supplement K	1000X	1X	20 µL	40 µL	100 µL

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 4 Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL	40 µL	100 µL
	9	Day 4 Supplement	1000X	1X	20 µL	40 µL	100 µL
	10	Astrocyte Supplement	1000X	1X	20 µL	40 µL	100 µL
	11	Supplement K	1000X	1X	20 µL	40 µL	100 µL

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Maintenance Medium	1	DMEM/F12 Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	2	Neurobasal Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 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.