BrainXell Neuron/Microglia Co-culture Assay Protocol (v11.0)

CONTENTS

- One vial of cryopreserved human iPSC-derived Neurons (multiple options)
- One vial of cryopreserved human iPSC-derived Microglia (BX-09XX-XX)
- BrainXell supplements:
    - BrainFast Motor #BX-2100 or BrainFast Glut #BX-2300 or BrainFast GABA #BX-2400
    - BrainFast D4 (BX-2040)
    - BrainFast SK (BX-2020)

SAFETY PRECAUTIONS

CAUTION: The use of full personal protective equipment (PPE) is strongly recommended when handling or transferring cryovials including safety-rated goggles, face shield, cryogenic gloves, and a lab coat. Any cryovial stored in liquid nitrogen has the potential to rapidly expand upon warming and carries the risk of rupture or explosion. Avoid submerging the vial cap in liquid nitrogen to reduce risk.

STORAGE

- Immediately transfer the cryovial of neurons to a liquid or vapor nitrogen storage system.
- BrainFast supplements should be stored at -20°C (6 months) or -80°C (18 months). Return vial to -20°C between each time of use to maintain stability.

ADDITIONAL MATERIALS NEEDED

- DMEM/F-12 Medium, +L-glutamine +HEPES (Thermo Fisher Scientific #11330032)
- Neurobasal Medium (Thermo Fisher Scientific #21103049)
- B-27 Supplement (Thermo Fisher Scientific #17504044)
- N-2 Supplement (Thermo Fisher Scientific #17502048)
- GlutaMAX Supplement (Thermo Fisher Scientific #35050061)
- Geltrex (Thermo Fisher Scientific #A1413201) or Cultrex (R&D Systems #3432-001-01)
- Chemically Defined Lipid Concentrate (Thermo Fisher Scientific #11905031)
- M-CSF (Thermo Fisher Scientific #300-25)
- IL-34 (Thermo Fisher Scientific #200-34)
- Ascorbic Acid (MilliporeSigma #A8960)
- PDL-Coated 96-Well Plates (Refer to BrainXell PDL Plate Coating Protocol)

OPTIONAL MATERIALS

Not included in medium during BrainXell QC assessment

- BDNF (Thermo Fisher Scientific #450-02)
- GDNF (Thermo Fisher Scientific #450-10)
- TGF-β1 (Thermo Fisher Scientific #100-21C)

PROCEDURE

Day 0 Seeding Preparation

1. Gather the Base Medium components according to the recipe (see Media Compositions section below).
    a. Note that BDNF, GDNF, IL-34, M-CSF, and TGF-β1 are lyophilized powders. Follow the manufacturer's instructions for reconstitution and long-term storage. We recommend creating stock solutions of 10 µg/mL BDNF, 10 µg/mL GDNF, and 2 µg/mL TGF-β1if they will be added to the culture medium.
    b. We recommend reconstituting ascorbic acid in DMEM/F-12 medium to create a 200 mM stock solution; See "Preparation of 200 mM Ascorbic Acid" at end of this document for important information.
2. Working in a cell culture hood (biological safety cabinet), combine all Base Medium components in an appropriately sized sterile container. Allow the Base Medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium in a 37°C water bath. Culture plates should also be at room temperature prior to use.
3. Prepare a pre-diluted Cultrex solution: add 495 µL of **cold** DMEM/F12 into a 55 µL aliquot of frozen Cultrex taken directly from the -80 freezer. Mix to dissolve and store at 4°C until it is time to seed the plate (Step 16).
4. Prepare one 50-mL conical tube: add 3 mL of Base Medium.
5. Prepare one microcentrifuge tube: add 25 µL of Trypan Blue solution for cell counting.

Day 0 Seeding the Neurons

6. Safety Reminder: the use of safety PPE is recommended during the handling of frozen cryovials. Remove the cryovial of cells from nitrogen storage and place in a 37°C water bath. To minimize contamination, avoid submerging the cap. Gently move the vial within the bath to increase the rate of thawing.
7. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovial from the water bath. Disinfect the vial by spraying it with 70% ethanol before transferring it into the cell culture hood.
8. Using a P1000 pipette, transfer 500 µL Base Medium from the prepared 50-mL conical tube to the cell cryovial at a rate of ~2-3 drops/sec. This process should take about 10 seconds.
9. Gently transfer all contents from the cryovial (~1mL total) back to the same 50-mL conical tube. Rinse the cryovial with 1 mL additional Base Medium to collect residual cells and transfer to the same 50-mL tube.
10. Centrifuge cells at 1700 rpm (465xg) for 5 mins.
11. Carefully aspirate supernatant, leaving the cell pellet, and resuspend cells in 950 µL fresh Base Medium. Mix thoroughly by gently pipetting up and down using a P1000 pipette.
12. Based on the CoA value (*Viable Cells/Vial*), resuspend the cells to a concentration of $1.0x10^6$ live cells/mL by slowly adding additional Base Medium to the existing ~1 mL in the tube.
    a. Example: 5.3 million Viable Cells/Vial is diluted to 5.3 mL total volume.
13. Count the cells: gently swirl the conical tube and pipette up and down 3-5 times to ensure cells are evenly suspended in medium. Transfer 25 µL of cell suspension to the microcentrifuge tube prefilled with 25 µL Trypan Blue solution from Step 5 and pipette up and down a few times to mix. Count the number of viable and dead cells using a hemocytometer. Determine the live cell concentration (live cells/mL) and viability.
14. Calculate volumes needed to make the motor neuron (MN) Seeding Suspension. A typical seeding density is 20,000 – 30,000 viable cells/100 µL/well for a 96-well plate (~62,500 – 93,750 viable cells/cm^2). Recommended seeding density may vary based on lot number; refer to the CoA for lot-specific seeding information. Dilute the cells to the desired seeding concentration based on the Trypan Blue cell count.

Example of dilution calculations.

Actual Live Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Cell Suspension**	**Volume of Base Medium**
Ex: 1.1x10^6 viable cells/mL *(Step 13)*	20,000 viable cells per 100 *µL/well* = *200,000 cells/mL*	11 mL *(Seeding 1 plate)*	**Mix: 2 mL** *(200,000/mL x 11mL) 1.1x10^6 cells/mL*	**Add: 9 mL** *(11mL – 2mL)*

15. In a new sterile 50-mL conical tube, mix the calculated volumes of cell suspension and Base Medium needed to obtain a final volume of 11 mL Neuron Seeding Suspension.
16. Add appropriate volumes of BrainFast Motor, Glut, or GABA based on neuron type (1:1000) and pre-diluted Cultrex from Step 3 (1:100) to the Neuron Seeding Suspension per the Seeding Medium recipe.
    b. Example: add 11 µL of BrainFast Motor and 110 µL pre-diluted Cultrex solution to 11 mL of Neuron Seeding Suspension for motor neurons.
17. Mix completely and transfer 100 µL/well (Ex: 20,000 cells/well) of the final Seeding Medium into a PDL-coated 96-well plate using a multi-channel pipette or liquid handler. Do not move or agitate the plate throughout the duration of the seeding process as this may lead to uneven attachment.
18. After seeding, let the plate rest for 10 minutes before moving it to allow the cells to settle to the bottom of the well. After 10 minutes, very gently transfer the plate to a humidified incubator at 37°C with 5% CO_2. The day of cell plating (today) is designated as Day 0.

**Note: The entire thawing and plating process should not exceed 1 hour. Post-thaw viability and overall cell health could be severely impacted and lead to an unsuccessful culture if the whole process is too long.*

Day 1 Medium Replacement

19. On Day 1 (24 hours after seeding), prepare fresh Day 1 Medium.
20. Carefully remove all 100 µL medium/well and gently replace with 100 µL/well Day 1 Medium. Complete one row or column at a time to ensure the wells do not dry out during the medium replacement process.

Day 4 Medium Addition

21. On Day 4 (96 hours after seeding), prepare fresh Day 4 Medium.
    c. Note: If only culturing for 7 days the addition of BrainFast SK on Day 4 is optional for motor neurons. If culturing longer than 7 days, the addition of BrainFast SK on Day 4 is recommended.
22. Gently add 100 µL/well of Day 4 medium to the entire plate for a total of 200 µL/well.

Day 7 Seeding of Microglia

23. Gather the components for the Day 7 MG Seeding Medium according to the recipe below and mix components in an appropriately sized sterile container. Allow the medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium in a 37°C water bath.
24. Prepare one 50-mL conical tube: add 3 mL of Day 7 MG Seeding Medium.
25. Prepare a microcentrifuge tube: add 25 µL of Trypan Blue solution for cell counting.
26. Remove the cryovial of microglia from liquid nitrogen storage and place in a 37°C water bath. To minimize contamination, avoid submerging the caps. Gently swirl the vials within the bath to increase the rate of thawing.
27. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovial from the water bath. Disinfect the vial by spraying it with 70% ethanol before transferring it into the cell culture hood.

28. Slowly add 500 µL of Day 7 MG Seeding Medium to the cryovial at a rate of ~2-3 drops/sec using a P1000 pipette. This process should take about 10 seconds.
29. Gently transfer all contents from the cryovial (~1mL total) to the prepared 50-mL conical tube. Rinse the cryovial with 1 mL additional MG Seeding Medium to collect residual cells and transfer to the 50-mL tube.
30. Centrifuge the tube of microglia at 1115 rpm (200xg) for 5 mins.
31. Carefully aspirate the supernatant, leaving the cell pellet, and resuspend cells in 950 µL fresh Day 7 MG Seeding Medium. Mix thoroughly by gently pipetting up and down using a P1000 pipette.
32. Based on the CoA value (*Viable Cells/Vial*), resuspend the cells to a concentration of $1.0x10^6$ live cells/mL by slowly adding additional Day 7 MG Seeding Medium to the existing ~1 mL in the tube.
    a. Ex: 2.3 million live cells per CoA will be resuspended to a <u>total</u> volume of 2.3 mL.
33. Count the cells: gently swirl the conical tube and pipette up and down 3-5 times to ensure cells are evenly suspended in medium. Transfer 25 µL of the microglia suspension to the microcentrifuge tube prefilled with 25 µL Trypan Blue solution and pipette up and down a few times to mix. Count the number of viable and dead cells using a hemocytometer. Determine the live cell concentration (live cells/mL) and viability. Remember to calculate in your dilution factor.
34. Calculate the volumes needed to make the final microglia seeding suspension. It is recommended to use a neuron:microglia ratio of 2:1 to 4:1, but your final ratios may vary depending on individual assay needs. Co-culture totals > 45,000 live cells/well may appear clustered due to the higher density.
    a. Dilute the cells in Day 7 MG Seeding Medium to the desired seeding concentration using the calculation table below as an example.

Example of cell dilution calculations for addition of microglia to (1) <u>96-well</u> plate.

Actual Live MG Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Microglia Suspension**	**Volume of MG Seeding Medium**
$1.1x10^6$ viable cells/mL *(Step 33)*	10,000 viable cells per 100 µL/well = *100,000 cells/mL*	11 mL *(Seeding 1 plate)*	**Add: 1 mL** *<u>100,000/mL x 11mL</u>* *$1.1x10^6$ cells/mL*	**Add: 10 mL** *(11mL – 1mL)*

35. Carefully remove 100 µL/well of spent medium from each well and add in 100 µL/well of microglia seeding suspension.
36. 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 9 and Onward Medium Changes

37. On Day 9 and onward, prepare Maintenance Medium according to the recipe below by adding the required reagents to room temp Base Medium stock.
38. Change half the medium (100 µL/well) twice weekly (on Day 9, 12, 15, etc.) using freshly prepared Maintenance Medium.
39. Co-cultures can be maintained viable and adherent in culture under the above conditions for at least 3 weeks post-seeding.

Co-Culture of Neurons/Microglia Media Compositions

Step 2		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Base Medium	1	DMEM/F12 Medium	1X	0.5X	48 mL	96 mL	240 mL
	2	Neurobasal Medium	1X	0.5X	48 mL	96 mL	240 mL
	3	B-27 Supplement	50X	1X	2 mL	4 mL	10 mL
	4	N-2 Supplement	100X	1X	1 mL	2 mL	5 mL
	5	GlutaMAX	200 mM	0.5 mM	250 µL	500 µL	1.25 mL
	6	Ascorbic Acid Solution (Step1b)	200 mM	0.2 mM	100 µL	200 µL	500 µL
		Base Medium stock can be stored for up to 3 weeks at 4°C.					

Note: Addition of Growth Factors is optional. If added, recommended final medium concentrations are 10 ng/mL BDNF, 10 ng/mL GDNF, and 1ng/mL TGF-β1.

Step 16		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Seeding Medium	1	**Neuron Seeding Suspension** (Step 15)	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast (Motor, Glut, or GABA)*	1000X	1X	11 µL	22 µL	55 µL
	3	Pre-diluted Cultrex**	1.5mg/mL	15 µg/mL	110 µL	220 µL	550 µL

* If seeding a mix of Cortical Glutamatergic and Cortical GABAergic neurons, use BrainFast Glut in place of BrainFast GABA.

** Cultrex must be pre-diluted according to step 3 in the protocol and added to the final seeding suspension right before seeding the plate.

Step 19		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 1 Medium	1	**Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast (Motor, Glut, or GABA)*	1000X	1X	11 µL	22 µL	55 µL
	3	BrainFast SK	1000X	1X	11 µL	22 µL	55 µL

* If seeding a mix of Cortical Glutamatergic and Cortical GABAergic neurons, use BrainFast Glut in place of BrainFast GABA.

Step 21		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 4 Medium	1	**Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast D4	1000X	1X	11 µL	22 µL	55 µL
	3	BrainFast SK***	1000X	1X	11 µL	22 µL	55 µL

*** On Day 4, BrainFast SK is recommended if co-cultures contain Cortical GABAergic and/or Spinal Motor neurons. Please contact support@brainxell.com if further assistance is needed.

Step 23		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 7MG Seeding Medium	1	**Base Medium**	**1X**	**1X**	**20 mL**	**40 mL**	**100 mL**
	2	Chemically Defined Lipid Mix	1X	0.005X	100 µL	200 µL	500 µL
	3	M-CSF	100 µg/mL	20 ng/mL	4 µL	8 µL	20 µL
	4	IL-34	100 µg/mL	100 ng/mL	20 µL	40 µL	100 µL

Step 37		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Maintenance Medium	1	**Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	Chemically Defined Lipid Mix	1X	0.005X	55 µL	110 µL	550 µL
	3	M-CSF	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL
	4	IL-34	100 µg/mL	100 ng/mL	11 µL	22 µL	55 µL

Preparation of 200 mM Ascorbic Acid Solution

Additional Sterile Materials Required

50-mL Conical Tubes, 0.22 micron Filter, Amber Microcentrifuge Tubes

Critical Information

- Ascorbic Acid must be added *into* the DMEM/F12 (not vice versa) to ensure it dissolves.
- The rate of Ascorbic Acid addition to DMEM/F12 is critical. If added too rapidly, the chemical will fail to go into solution.
- Only mix by inversion. Undissolved Ascorbic Acid will adhere to pipette tips and other implements.
- Ascorbic Acid is pH, temperature, and light sensitive.

Steps

1. Aliquot 17.2 mL of DMEM/F12 into a 50-ml tube.
2. Weigh 1.0 gram of Ascorbic Acid using a separate vessel.
3. **Critical Step**: Gradually transfer Ascorbic Acid ***into*** the tube of DMEM/F12 (no more than 0.2 gram at a time, waiting 10 seconds between each addition).
4. Secure the cap on the tube and invert to mix.
5. Allow the Ascorbic Acid to dissolve into the solution:

a. Place in a 37°C water bath for 30 minutes, inverting the tube every 10 minutes to mix.

b. After 30 minutes check to see if Ascorbic Acid is fully dissolved. If not, invert tube to mix and place capped tube at 4°C overnight.
c. After overnight storage, invert to mix and confirm Ascorbic Acid is completely dissolved.
6. Filter the fully dissolved Ascorbic Acid Solution using a Steriflip filter or equivalent 0.22 µm sterile filter.
7. Aliquot into amber 1.5mL Eppendorf tubes; recommended volumes of 110µl or 550µl.
8. Maintain frozen aliquots at -20°C for up to 6 months. Aliquots are single use. Do not refreeze.