OPC Maturation with Neurons 96-well Co-culture Protocol (vb.2)

BACKGROUND

BrainXell's Oligodendrocyte Progenitor Cells (OPC) will mature when cultured with BrainXell iPSC-derived neurons, resulting in higher expression of myelin basic protein.

CONTENTS

- One vial of cryopreserved human iPSC-derived OPCs (BX-1300, BX-13XX-XX)
- One vial of cryopreserved human iPSC-derived Spinal Motor, Cortical Glutamatergic or GABAergic Neurons (BX-01XX-XX, BX-03XX-XX, or BX-04XX-XX)
- BrainXell supplements:
    - BrainFast OPC (BX-3300)
    - BrainFast included with neurons is based on neuronal subtype.
        - 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

- Cryovials of cells should immediately be transferred to a liquid or vapor nitrogen storage system upon receipt of shipment.
- BrainFast supplements should be stored at -20°C.
    - Supplements should be kept at -20°C between each time of use to maintain stability. See label for expiry.

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)
- Cultrex (R&D Systems #3432-001-01)
- Ascorbic Acid (MilliporeSigma #A8960)
- BDNF (Thermo Fisher Scientific #450-02)
- Doxycycline Hyclate (Sigma #D9891)
- iMatrix511(Nacalai #892021)

- PDL-Coated 96-Well Plates (Refer to BrainXell PDL Plate Coating Protocol)
- If culturing spheroids, we recommend either PrimeSurface 3D Culture Spheroid (ULA) plates (S-Bio #MS-9096WZ) or Nunclon Sphera 96-Well U-Shaped-Bottom Microplate (Thermo Fisher Scientific #174925).

PROCEDURE

Important: this procedure is specific to the cell types listed above. To incorporate other cell types into the culture, please reach out to support@brainxell.com for additional information.

Day 0 Seeding Preparation

For a 96-well plate, a final combined seeding density of 40,000 – 45,000 viable cells per well is recommended (~125,000 – 140,625 viable cells/cm^2). Seeding ratios of 1:2 have been shown to work well for neuron:OPC cultures. Tri-culture totals > 50,000 live cells/well in a 96-well plate may appear clustered due to the higher density.

1. Gather the Base Medium components according to the recipe (see Media Compositions section below).
    a. Note that BDNF and doxycycline are lyophilized powders. Follow the manufacturer's instructions for reconstitution and long-term storage.
    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 18).
4. Prepare one 50-mL conical tube for the neurons: add 3 mL of Base Medium.
5. Prepare one microcentrifuge tube for the neurons: add 25 µL of Trypan Blue solution for cell counting.

Day 0 Seeding of Neurons

A general recommended seeding ratio of neurons to OPCs 1:2. Other ratios may be suitable depending on individual research needs.

Safety Reminder: the use of safety PPE is recommended during the handling of frozen cryovials.

6. Remove the cryovial of neurons 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.
7. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovials from the water bath. Disinfect the vials by spraying them with 70% ethanol before transferring them to the cell culture hood.
8. Using a P1000 pipette, transfer 500 µL of 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 per cryovial.
9. Gently transfer all contents from the thawed neuron cryovial (~1mL total) back to the same labeled 50-mL conical tube. Add 1 mL of additional Base Medium to the cryovial to collect any residual cells and transfer into the same 50-mL tube of neurons.

10. Centrifuge the tube of neurons 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 until no clumps are visible.
12. Based on the number of *Viable Cells/Vial* listed on the Certificate of Analysis (CoA), further dilute the cells in the 50-mL tubes to reach a concentration of $1.0x10^6$ live cells/mL by slowly adding additional Base Medium to the tube.
    a. Ex: If the CoA shows 6.7 million live cells/vial, (1) vial will be resuspended to a final volume of 6.7 mL or (2) combined vials will be diluted to 13.4 mL.
13. Count the resuspended cells in the tube: 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 cell suspension to the matching labeled 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 of the cell suspension.
14. Calculate the volume of cell suspension needed to make the final seeding suspension. For a 96-well plate, a seeding density of 15,000 – 20,000 viable neurons per well is recommended (~46,875 – 62,500 viable cells/cm^2). Dilute the cells to the desired seeding concentration using the calculation table below as an example. Other seeding densities may be suitable depending on user preference.

    Example calculations for seeding (1) 96-well plate of neurons at 20,000 cells/100 µL/well:

Actual Neuron Live Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Cell Suspension**	**Volume of Base Medium**
$1.1x10^6$ viable cells/mL *(Step 13)*	20,000 viable cells per 100 µL/well = *200,000/mL*	11 mL *(Seeding 1 plate)*	**Mix: 2 mL** *(200,000/mL x 11mL) / 1.1x10⁶ cells/mL*	**Add: 9 mL** *(11mL – 2mL)*

15. In a new sterile 50-mL tube: add the calculated volume of cell suspension and add enough Base Medium to reach 11 mL total cell suspension (per plate).
16. Per the Day 0 Neuron Seeding Suspension recipe, add in the appropriate volumes of the required BrainFast supplement and pre-diluted Cultrex from Step 3 (1:100).
    a. Example: add 11 µL of BrainFast Motor and 110 µL pre-diluted Cultrex solution to 11 mL total of neuron cell suspension.
17. Using a 5-mL serological pipette, slowly mix the suspension to ensure a homogeneous suspension.
18. Seed 100 µL/well of the Day 0 Neuron Seeding Suspension onto a PDL-coated 96-well plate using a multi-channel pipette or liquid handler. Throughout the seeding process, be careful not to move or agitate the plate as this may lead to uneven attachment.
19. 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 OPC Seeding into Established Neuron Wells

20. Bring Base Medium to room temperature but leave the prior seeded neuron plate in the incubator until you are ready to add the OPCs to the wells.
21. Prepare one 50-mL conical tube: add 3 mL of Base Medium.
22. Prepare one microcentrifuge tube: add 25 µL of Trypan Blue solution for cell counting.

Safety Reminder: the use of safety PPE is recommended during the handling of frozen cryovials.

23. Remove the cryovial of OPCs 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.
24. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovials from the water bath. Disinfect the vials by spraying them with 70% ethanol before transferring them to the cell culture hood.
25. Using a P1000 pipette, transfer 500 µL of 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 per cryovial.
26. Gently transfer all contents from the thawed OPC cryovial (~1mL total) back to the same labeled 50-mL conical tube. Add 1 mL of additional Base Medium to the cryovial to collect any residual cells and transfer into the same 50-mL tube of OPCs.
27. Centrifuge the tube of OPCs at 1700 rpm (465xg) for 5 mins.
28. 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 until no clumps are visible.
29. Based on the number of *Viable Cells/Vial* listed on the Certificate of Analysis (CoA), further dilute the cells in the 50-mL tubes to reach a concentration of $1.0x10^6$ live cells/mL by slowly adding additional Base Medium to the tube.
    a. Example: If the CoA shows 5.3 million live cells/vial, dilute to a final volume of 5.3 mL.
30. Count the resuspended cells in the tube: 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 cell suspension to the matching labeled 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 of the cell suspension.
31. Calculate the volume of cell suspension needed to make the final seeding suspension. For a 96-well plate, a seeding density of 20,000 – 30,000 viable OPCs per well is recommended (62,500 – 93,750 viable cells/cm^2) to be added to the established neuron wells. Dilute the OPCs to the desired seeding concentration using the calculation table below as an example. Other seeding densities may be suitable depending on user preference.

    Example calculations for seeding (1) 96-well plate of OPCs at 20,000 cells/50 µL/well:

Actual OPC Live Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Cell Suspension**	**Volume of Base Medium**
$1.1x10^6$ viable cells/mL *(Step 30)*	20,000 viable cells per 50 µL/well = *400,000/mL*	*5.5 mL (Seeding 1 plate)*	**Mix: 2 mL** *(400,000/mL x 5.5mL) / $1.1x10^6$ cells/mL*	**Add: 3.5 mL** *(5.5mL – 2mL)*

32. In a new sterile 50-mL tube: add the calculated volume of cell suspension and add enough Base Medium to reach 5.5 mL total cell suspension (per plate).
33. Per the Day 1 OPC Seeding Medium recipe, add in the appropriate volumes of the required BrainFast OPC and BrainFast neuronal supplements and the additional required components into the cell suspension.
    b. Example: add iMatrix 511 (110 µL), BrainFast OPC (11 µL), doxycycline (11 µL), and BrainFast Glut or other neuron type (5.5 µL) into 5.5 mL of cell suspension.
34. Remove the seeded neuron plate from the incubator and remove half the medium from the existing wells (remove 50 µL/well) to prepare for seeding the OPCs.
35. Using a 5-mL serological pipette, slowly pipette the 50-mL tube containing the OPCs to ensure a homogeneous suspension before seeding.

36. Using a multi-channel pipette or liquid handler, seed 50 µL/well of the OPCs resuspended in Day 1 OPC Seeding Medium into the neuron wells. Throughout the seeding process, be careful not to move or agitate the plate as this may lead to uneven OPC attachment. The final combined well volume is 100 µL/well.
37. After seeding, let the plate rest for 10 minutes before moving it to allow the OPCs 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 OPC plating (today) is designated as Day 1.

Day 2 Medium Addition

38. On Day 2 (48hrs after neuron seeding/24hrs after OPC seeding), prepare fresh Day 2 Medium according to the recipe below by adding the required reagents to room temp Base Medium.
39. Carefully add 100 µL/well of freshly prepared room temp Day 2 Medium to the entire plate. Final well volume is 200 µL/well.

Day 3 Half Medium Replacement

40. On Day 3 (72hrs after neuron seeding/48hrs after OPC seeding), prepare fresh Day 3 Medium according to the recipe below by adding the required reagents to room temp Base Medium.
41. Carefully remove 100 µL/well of medium and replace with 100 µL/well of fresh Day 3 Medium.

Day 6 and Onward Half Medium Replacements

42. On Day 6 and onward, prepare fresh OPC/Neuron Maintenance Medium, according to the recipe below by adding the required reagents to room temp Base Medium.
43. Change half the medium (100 µL/well) twice weekly (on Day 6, 10, 13, etc.) using freshly prepared OPC/Neuron Maintenance Medium.

Co-cultures can be maintained viable and adherent in culture under the above conditions for up to two weeks.

OPC/Neuron Co-Culture 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.						

Step 16		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 0 Neuron Seeding Suspension	1	**Neuron Cell 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**	1.5 mg/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. For other neuron types contact support@brainxell.com for supplement and necessary medium composition recommendations.

** 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 33		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 1 OPC Seeding Medium		**OPC Suspension** (Step 32)	**1X**	**1X**	**5.5 mL**	**11 mL**	**27.5 mL**
	1	iMatrix511	100X	2X	110 µL	220 µL	550 µL
	2	BrainFast OPC	1000X	2X	11 µL	22 µL	55 µL
	3	Doxycycline	2 mg/mL	4 µg/mL	11 µL	22 µL	55 µL
	4	BrainFast (Motor, Glut, or GABA)*	1000X	1X	5.5 µL	11 µL	27.5 µL

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

Step 38		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 2 Medium	1	**Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BDNF	10 µg/mL	20 ng/mL	22 µL	44 µL	110 µL
	3	BrainFast SK	1000X	2X	22 µL	44 µL	110 µL
	4	BrainFast (Motor, Glut, or GABA)*	1000X	1X	11 µL	22 µL	55 µL
	5	BrainFast OPC	1000X	1X	11 µL	22 µL	55 µL
	6	Doxycycline	2 mg/mL	2 µg/mL	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 40		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 3 Medium	1	**Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BDNF	10 µg/mL	10 ng/mL	11 µL	22 µL	55 µL
	3	BrainFast OPC	1000X	1X	11 µL	22 µL	55 µL
	4	Doxycycline	2 mg/mL	2 µg/mL	11 µL	22 µL	55 µL
	5	BrainFast D4	1000X	1X	11 µL	22 µL	55 µL

Step 42		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
OPC/Neuron Maintenance	1	**Base Medium**	**1X**	**1X**	**22 mL**	**44 mL**	**110 mL**
	2	BDNF	10 µg/mL	10 ng/mL	22 µL	44 µL	110 µL
	3	BrainFast OPC	1000X	2X	44 µL	88 µL	220 µL
	4	Doxycycline	2 mg/mL	4 µg/mL	44 µL	88 µL	220 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.