BrainXell OPC Proliferative Monoculture

Protocol (vb.3-1)

BACKGROUND

BrainXell's Oligodendrocyte Progenitor Cells (OPC) are proliferative in vitro. This protocol allows OPC cells to remain in a proliferative state for 7-10 days for OPC research needs. If a mature oligodendrocyte with higher expression of myelin basic protein is desired, OPCs should be seeded following the BrainXell OPC Maturation with Neurons 96-well Co-culture protocol.

CONTENTS

- One vial of cryopreserved human oligodendrocyte precursor cells (BrainXell BX-1300, BX-13XX-XX)

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 cryovials of cells to a liquid or vapor nitrogen storage system.

ADDITIONAL MATERIALS NEEDED

- DMEM/F-12 Medium, +L-glutamine +HEPES (Thermo Fisher Scientific #11330032)
- N-2 Supplement (Thermo Fisher Scientific #17502048)
- GlutaMAX Supplement (Thermo Fisher Scientific #35050061)
- Ascorbic Acid (MilliporeSigma #A8960)
- Doxycycline Hyclate (Sigma #D9891)
- Heparin (MilliporeSigma #H3149)
- FGF-8b (Thermo Fisher Scientific #AF-100-25)
- HGF (Thermo Fisher Scientific #100-39)
- NT-3 (Thermo Fisher Scientific #450-03)
- PDGF-AA (Thermo Fisher Scientific #100-13A)
- Cultrex (R&D Systems #3432-001-01)
- 96-Well TC-treated Plates (Revvity #6055302)
    - Note: plates will be manually precoated in Step 3 before seeding

PROCEDURE

Seeding and Plate Preparation

To seed (1) full 96-well plate you will need approximately 2.7-3 million live cells. A targeted seeding density of 78,125 to 93,750 live cells/cm^2 can be used to calculate the number of live OPCs needed to seed other plate types. Additional cells may be used depending on individual research needs. Review the Certificate of Analysis (CoA) for viability and seeding information.

1. Gather the Base Medium components according to the recipe (see Media Compositions section below).
    a. Note that doxycycline, heparin, FGF-8, HGF, NT-3, and PDGF-AA 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 OPC Proliferation Base Medium components in an appropriately sized sterile container.
3. Important: Plates must be coated with Cultrex at least two hours before seeding. The Cultrex coating solution must be prepared with **cold** OPC Proliferation Base Medium.
    a. To coat (1) 96-well plate, add 450 µL of **cold** OPC Proliferation Base Medium to a 50 µL frozen aliquot of Cultrex and place at 4°C until fully thawed.
    b. Once the Cultrex is thawed, add 7 mL of **cold** Base Medium to a conical tube and thoroughly mix in the 500 µL of thawed, prediluted Cultrex solution.
        i. Using a multichannel pipette, immediately transfer the prepared **cold** Cultrex medium to the 96-well TC plate (50 µL/well). Verify that all well bottom are completely covered with medium. Gently tap or swirl the plate to disperse the solution if needed.
    c. Place the Cultrex-coated plate in a 37°C incubator for a minimum of 2 hours prior to seeding. Overnight incubation at 4°C is suitable for next day seeding. Note: The Cultrex solution will remain in the well for seeding.

Day 0 Seeding the OPCs

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

4. Allow the OPC Proliferation Base Medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium to 37°C.
5. Transfer the precoated Cultrex plate from the incubator or fridge to the BSC in preparation for seeding. The Cultrex solution in the wells should be room temp prior to seeding.
6. Remove the cryovial of cells from nitrogen storage and place the cryovial of cells 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. Prepare one 50-mL conical tube: add 3 mL of OPC Proliferation Base Medium that will be used to dilute the thawed cells.
8. Prepare one microcentrifuge tube: add 25 µL of Trypan Blue solution for cell counting.
9. 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.
10. Using a P1000 pipette, transfer 500 µL OPC Proliferation Base Medium from the 50-mL conical tube to the cell cryovial at a rate of ~2-3 drops/sec. This process should take about 10 seconds.
11. Gently transfer all contents from the cryovial (~1mL total) back to the same labeled 50-mL conical tube.
12. Add 1 mL of additional OPC Proliferation Base Medium to the cryovial to collect any residual cells and transfer to the same 50-mL tube.
13. Centrifuge cells at 1700 rpm (465xg) for 5 mins.

14. Carefully aspirate supernatant, leaving the cell pellet, and resuspend cells in 950 µL fresh OPC Proliferation Base Medium. Mix thoroughly, gently pipetting up and down using a P1000 pipette.
15. 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 OPC Proliferation Base Medium to the existing ~1 mL in the tube.
    a. Example: 5.3 million Viable Cells/Vial is diluted to 5.3 mL <u>total</u> volume.
16. 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 8 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.
17. Calculate volumes needed to make the OPC Seeding Suspension. A typical seeding density is 25,000-30,000 viable cells/50 µL/well for a 96-well plate (78,125 to 93,750 live cells/cm^2). Recommended seeding density may vary based on assay. Dilute the cells to the desired seeding concentration based on the viable 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 16**)*	30,000 viable cells per 50 µL/well = *600,000 cells/mL*	*5.5* mL *(Seeding 1 plate)*	**Mix: 3 mL** *(<u>600,000/mL x 5.5mL</u>) 1.1x10^6 cells/mL*	**Add: 2.5 mL** *(5.5mL –3mL)*

18. In a new sterile 50-mL conical tube, mix the calculated volumes of cell suspension and OPC Proliferation Base Medium needed to obtain a final volume of 5.5 mL OPC Seeding Suspension.
19. Add the appropriate required volumes of all additional reagents (doxycycline, heparin, FGF-8, HGF, NT-3, and PDGF-AA) as listed in the OPC Seeding Medium table.
20. Thoroughly, but gently, mix the final OPC Seeding Medium containing the cells and transfer 50 µL/well (Ex: 30,000 cells/well) directly into the Cultrex-filled wells of the 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.
21. 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 Addition

22. On Day 1 (24 hours after seeding), prepare fresh OPC Day 1 Medium.
23. Gently add 100 µL/well of fresh OPC Day 1 Medium for a total volume of 200 µL/well. Do not remove any media from the wells as the cells are still fragile at this point.

Day 3 Half Medium Replacement

24. On Day 3 (72 hours after seeding), prepare fresh OPC Maintenance Medium.
25. Carefully remove half the medium from the wells (100 µL/well) and gently add back fresh OPC Maintenance Medium (100 µL/well).

Day 6 Half Medium Replacement

26. On Day 6, prepare fresh OPC Maintenance Medium.
27. Carefully remove half the medium from the wells (100 µL/well) and gently replace with fresh OPC Maintenance Medium.

Day 7-10 Maintenance or Assay Completion

Note: OPC monocultures will stay attached to the plate and have typical morphology for up to 7-10 days. After this time, they may detach or begin to die off. To reach a mature oligodendrocyte phenotype and high MBP expression, OPCs will need to be seeded with neurons.

28. On Day 8 or 9, prepare fresh OPC Maintenance Medium.
29. Carefully remove half the medium from the wells (100 µL/well) and gently replace with fresh OPC Proliferation Maintenance Medium.

OPC monocultures are intended to be maintained up to a maximum of ten days after thaw. See BrainXell Protocols: OPC Maturation with Neurons 96-well Co-culture and O4 Immunostaining of OPCs for further information.

For further assistance email Support@brainxell.com.

OPC Proliferation Media Compositions

Step 2		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
OPC Proliferation Base Medium	1	DMEM/F12 Medium	1X	1X	100 mL	200 mL	500 mL
	2	N-2 Supplement	100X	1X	1 mL	2 mL	5 mL
	3	GlutaMAX	200 mM	0.5 mM	250 µL	500 µL	1.25 mL
	4	Ascorbic Acid	200 mM	0.2 mM	110 µL	220 µL	550 µL
		Base Medium stock can be stored for up to 3 weeks at 4°C.					

Step 19		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
OPC Seeding Medium	1	**OPC Seeding Suspension** (Step 18)	**1X**	**1X**	**5.5 mL**	**11 mL**	**27.5 mL**
	2	Doxycycline	2 mg/mL	4 µg/mL	11 µL	22 µL	55 µL
	3	Heparin	2 mg/mL	4 µg/mL	11 µL	22 µL	55 µL
	4	FGF-8b	100 µg/mL	40 ng/mL	2.2 µL	4.4 µL	11 µL
	5	HGF	100 µg/mL	40 ng/mL	2.2 µL	4.4 µL	11 µL
	6	NT-3	100 µg/mL	40 ng/mL	2.2 µL	4.4 µL	11 µL
	7	PDGF-AA	100 µg/mL	40 ng/mL	2.2 µL	4.4 µL	11 µL

Step 22		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
OPC Day 1 Medium	1	**OPC Proliferation Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	Doxycycline	2 mg/mL	2 µg/mL	11 µL	22 µL	55 µL
	3	Heparin	2 mg/mL	2 µg/mL	11 µL	22 µL	55 µL
	4	FGF-8b	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL
	5	HGF	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL
	6	NT-3	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL
	7	PDGF-AA	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
OPC Maintenance Medium	1	**OPC Proliferation Base Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	Doxycycline	2 mg/mL	4 µg/mL	22 µL	44 µL	110 µL
	3	Heparin	2 mg/mL	4 µg/mL	22 µL	44 µL	110 µL
	4	FGF-8b	100 µg/mL	40 ng/mL	4.4 µL	8.8 µL	22 µL
	5	HGF	100 µg/mL	40 ng/mL	4.4 µL	8.8 µL	22 µL
	6	NT-3	100 µg/mL	40 ng/mL	4.4 µL	8.8 µL	22 µL
	7	PDGF-AA	100 µg/mL	40 ng/mL	4.4 µL	8.8 µL	22 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.