

Protocol for the Co-culture of iPSC-derived Microglia with iPSC-derived Cerebral Cortical Neural Culture

Required reagents

Product code	Description
ax0031a + b	Neural Maintenance Medium + Supplement
ax0041	SureBond
ax0053	SureBond-XF
ax0047	Recombinant Human FGF2
ax68168 (5 mg)	Y-27632 Hydrochloride (ROCK Inhibitor)
ax0660	Microglia Maintenance Medium
ax0016, ax0111 (AD APOE4/4) or ax0112 (AD L286V)	Human iPSC-derived Cortical Neural Stem Cells (NSCs)
ax1666	Human iPSC-Derived Microglia Precursors

Stage One: Preparing the Cortical Neural Culture

Day -1: Coat a T25 cell culture flask with SureBond

- Upon receipt store SureBond at or below -80°C
- Dilute the SureBond stock solution (100x) 1:100 in 1 x PBS to make 1 x working solution e.g. 60 µL in 6 mL.
- Coat the surface of a T25 flask with the SureBond 1 x working solution. We recommend coating at a volume of 200 µL per cm².
- Incubate for overnight at 37°C.
- Remove the SureBond from the culture dish prior to seeding. Do not wash the culture vessel after coating with SureBond.
- Do not let the SureBond coating dry out before seeding the cells.
- Do NOT use SureBond-XF as a coating at this stage as it will not allow passaging.

Day -1: Prepare the Neural Maintenance Medium

- To prepare Complete Neural Maintenance Medium, thaw Neural Maintenance Supplement (ax0031a) overnight at 4°C. Add 7.5 mL of the thawed supplement to the entire 500 mL Neural Maintenance Basal Medium (ax0031b) and mix well.
- Once supplemented, it can be stored at 4°C for two weeks.

Day 0: Thaw Human iPSC-derived Neural Stem Cells

- Pre-warm all media and culture vessels to 37°C before use.
- To thaw cells – transfer the cells from storage by transporting cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a 37°C water bath.
- Quickly thaw the vial of cells in a 37°C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). After ~1-2 minutes, remove the vial before the last

- bit of ice has melted.
- Do not shake the vial during thawing.
 - Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with clean paper towel before placing the vial in the hood.
 - Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add 10 mL of pre-warmed, 37°C, Neural Maintenance Medium. Gently wash the cryogenic vial with 1 mL of Neural Maintenance Medium. Transfer this to the 15 mL sterile conical tube containing the cells.
 - Centrifuge cells at 200 x *g* for 5 minutes at room temperature.
 - During the centrifugation step, remove the coating solution.
 - Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in **1 mL** of Neural Maintenance Medium supplemented with 20 ng/mL FGF2 and 10 μM ROCK inhibitor until they are in a single cell suspension.
 - **Perform a cell count to ensure optimal seeding density.**
 - Plate the resuspended cells drop-wise and evenly at a density of **100,000 cells/cm²**
 - To ensure an even plating of Human iPSC-derived NSCs, gently rock the T25 flask back and forth and side to side several times.
 - Incubate the cells at 37°C, 5% CO₂.
 - **Critical step: Handle cells gently and as described or the viability will be low post-thaw.**

Day 1: Full medium change with Neural Maintenance Medium supplemented with 20 ng/mL FGF2

Day 3: Full medium change with Neural Maintenance Medium supplemented with 20 ng/mL FGF2

Day 5: Full medium change with Neural Maintenance Medium supplemented with 20 ng/mL FGF2

Day 6: Coat a fresh culture vessel with SureBond-XF

- Upon receipt store the SureBond-XF coating solution at 4°C.
- Dilute the SureBond-XF stock solution 1:200 in 1 x PBS to make 1 x working solution e.g. 75 μL in 15 mL.
- Coat the surface with the SureBond-XF 1 x working solution. We recommend coating at a volume of 150 μL per well for a 96-well plate
- Incubate overnight at 37°C.

Day 7: Passage the NSC culture to final culture format

- Rinse the T25 flask with iPSC-derived NSCs with 1x PBS.
- Add 1 mL per 10cm² Unlock (2.5 mL of unlock for a T25 flask) incubate 10 minutes at 37°C
- Add gently four volumes of pre-warmed, 37°C, Neural Maintenance Medium, (for example if 2.5 mL of Unlock is used, then add 10 mL of the medium to stop the reaction) pipette up and down 3 times.

- Centrifuge at 200 *xg* for 5 minutes
- Resuspend cells in Neural Maintenance Medium, plate at 100,000 cells/cm² onto SureBond-XF coated fresh tissue culture vessel.
- Do NOT remove the SureBond-XF from the tissue culture vessel prior to seeding. Do not wash the culture vessel after coating with SureBond-XF.
- Do not let the SureBond coating dry out before seeding the cells.

Day 9: Half medium change with Neural Maintenance Medium

Day 11: Half medium change with Neural Maintenance Medium

Day 13: Half medium change with Neural Maintenance Medium

Day 15: Half medium change with Neural Maintenance Medium

Day 17: Half medium change with Neural Maintenance Medium

Day 19: Half medium change with Neural Maintenance Medium

Stage Two: Preparing the Co-Culture

Day 21: Adding iPSC-derived Microglia to create co-culture

- Supplement the Microglia Maintenance Medium with Supplements A, B and C in the ratios displayed in the table below.

Component	Product Code	Stock Concentration	Final Concentration	For 50 mL Microglia Maintenance Medium
Microglia Maintenance Medium (Basal)	ax0660d	1X	1X	49.4 mL
Supplement A	ax0660a	10 µg/mL	10 ng/mL	50 µL
Supplement B	ax0660b	100 µg/mL	100 ng/mL	50 µL
Supplement C	ax0660c	100X	1X	500 µL

- Human iPSC-derived Microglia Precursors will arrive warm at ~37°C in a cryovial.
- Gently pipette the microglia to get them into suspension and transfer the cells to a 15 mL conical tube (~ 2.4 mL).
- Wash the vial **twice** with 1 mL **room temperature** supplemented Microglia Maintenance Medium each time
- Add a further 7.6 mL **room temperature** Microglia Maintenance Medium to the 15 mL conical tube to make up to 10 mL.
- Centrifuge the cells at **300 x g** for 5 minutes.

- Remove and discard the supernatant. Resuspend the cell pellet in **room temperature** Microglia Maintenance Medium.
- Perform a cell count to determine total number of viable microglia precursors
- Remove and discard the medium from the cortical neural culture.
- Gently add 100,000/cm² iPSC-derived Microglial precursors in Microglia Maintenance Medium to the neurons (We recommend 200 μ L of cell-medium mixture to be added to each well of a 96 well plate). Higher volume of medium is used because of the high density of cells (100,000 per cm² of neurons with 100,000 per cm² of microglia).

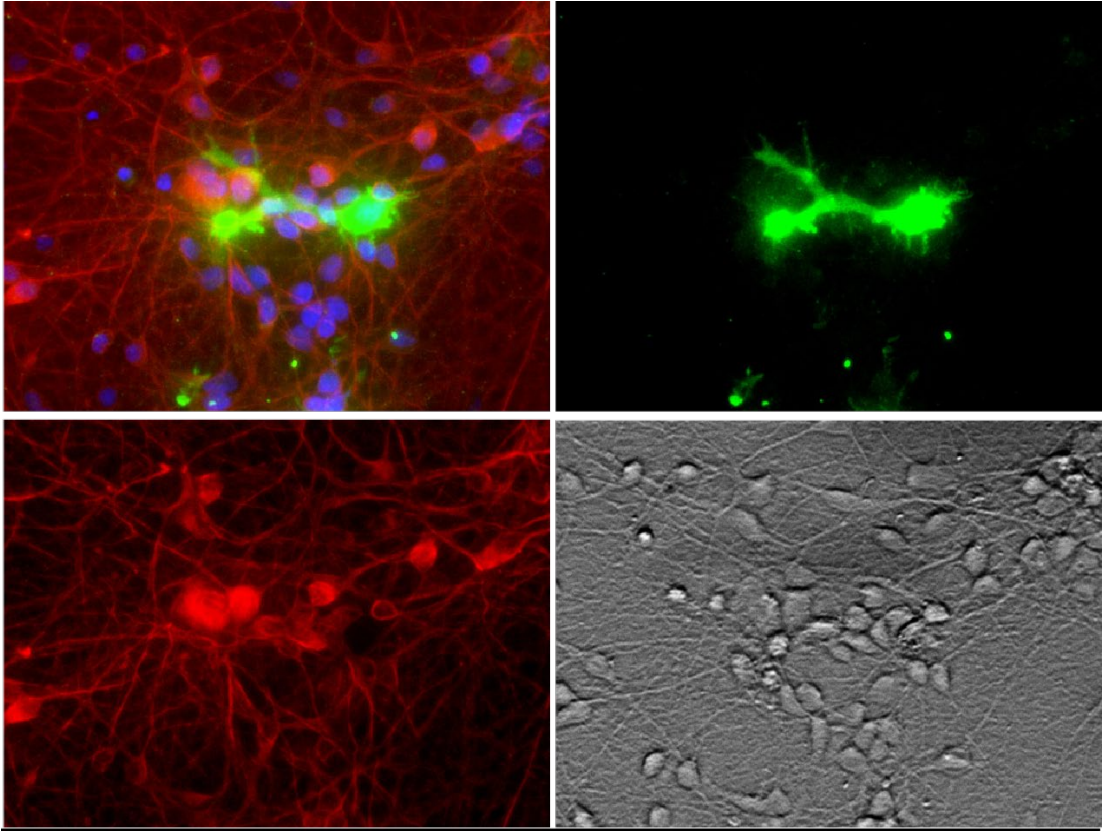
Day 22: Perform 2/3 medium change with Microglia Maintenance Medium.

Day 23: Perform half medium change with Microglia Maintenance Medium.

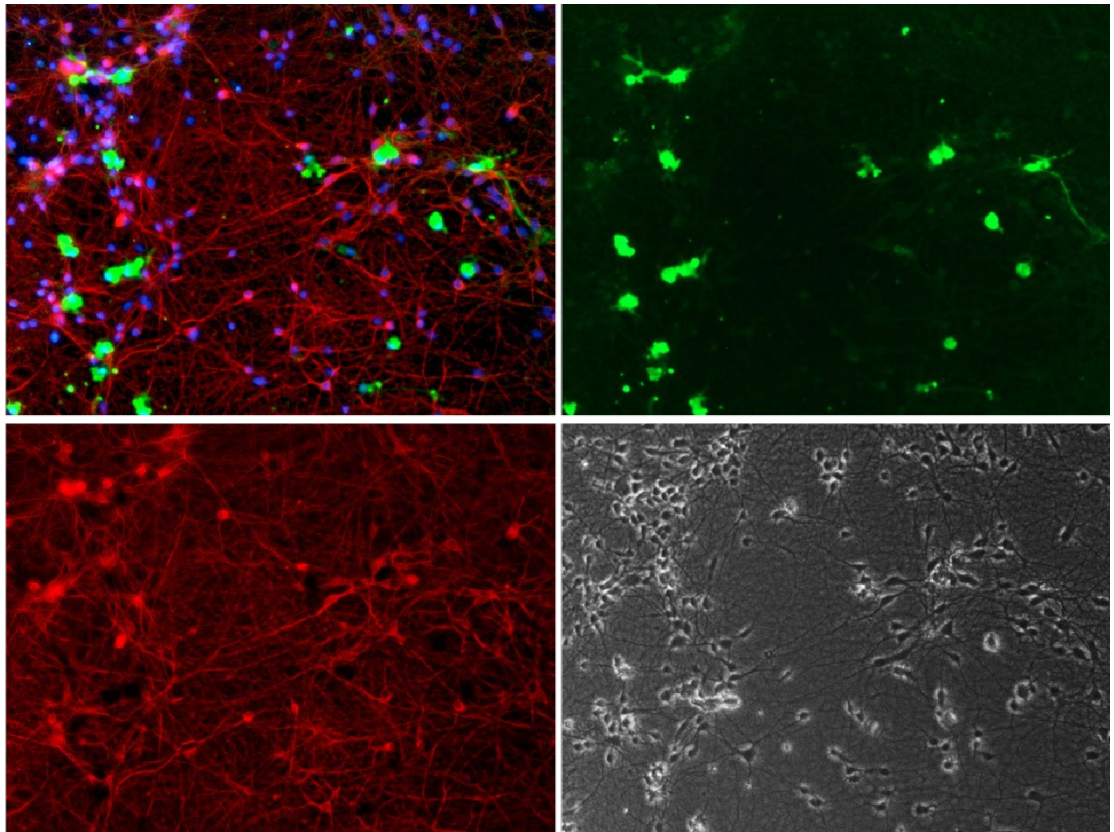
Repeat half medium change every other day until Day 35, at which the co-culture is ready for experiments. Optimally, assays should be run between Day 35 and Day 42

Representative images of the co-culture

IBA (G) TUJ1 (R) DAPI (B) 60X



IBA (G) TUJ1 (R) DAPI (B) 20X



Notes

In co-culture, neurons and microglia survive for at least 2 months without obvious proliferation of microglia. However, medium change can slowly deplete microglia, and neurons continue to proliferate, so the ratio changes over time. The culture will become very thick over extended timeframes, through neuronal proliferation, which can improve microglial ramification (which is greater the more the microglia are integrated into a dense neuronal environment), but will make imaging challenging.

Troubleshooting

Loss of microglia over the two weeks of co-culture: A proportion of the microglia will be loosely attached to the surface of the neurons at any one time, so it is important to conduct the medium changes in a way that minimises the risk of aspirating them, otherwise you will end up with very few microglia at the end of the experiment, and likely a variable number which would ruin any assay or experiment. So aspirate gently and slowly, without having jiggled the plate and without touching the bottom of the plate. Never exchange 100% of the medium, but 1/2 to 2/3 only.

Inconsistent results from 96 well plates: Because of evaporation issues over extended culture times, it is best to design the plate layout to avoid using the outer wells of 96 well plates. Corner wells are particularly susceptible to evaporation.