Tri-Culture of iCell Microglia with iCell Neuronal Cell Types and iCell Astrocytes

Introduction

Microglia are immune cells resident in the central nervous system responsible for fundamental physiological and pathological processes. Microglia support neuronal homeostasis and facilitate neuronal network formation by process surveillance and synaptic pruning. To study these important interactions *in vitro*, cell culture conditions which support the survival of multiple cell types simultaneously must first be established.

iCell® Microglia are derived from human induced pluripotent stem cells (iPSC) and express relevant microglial markers, such as TREM2, CX3CR1, TMEM119, P2RY12, and IBA1. Furthermore, these cells are fully differentiated and exhibit functionality similar to primary microglia, including phagocytosis and cytokine-mediated neuroinflammatory response. iCell Microglia are a consistent source of high-quality cells shipped cryopreserved and ready-to-use in combination with other iPSC-derived cell types.

This application protocol describes how to establish a neural tri-culture system with human iPSC-derived cells, featuring iCell Microglia, iCell Astrocytes and either iCell GABANeurons (GABAergic/inhibitory cortical neurons) or iCell GlutaNeurons (glutamatergic/excitatory cortical neurons). This tri-culture system may be used to study neuroinflammation, develop assays for neuroprotection or synaptic pruning, or support drug discovery efforts focused on neurodegenerative diseases, including Alzheimer's Disease.

Required Materials

The following materials are required in addition to the items specified in the iCell Microglia Quick Guide.

Item	Vendor	Catalog No.
Cells		
iCell Microglia Kit, 01279	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1131
• iCell Microglia, 01279	FUJIFILM Cellular Dynamics, Inc. (FCDI)	C1110
• iCell Glial Base Medium, 50 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1034
• iCell Microglia Supplement A (100X), 0.5 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1035
• iCell Microglia Supplement B (100X), 0.5 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1036
• iCell Neural Supplement C (25X), 2 x 1 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1037
iCell Astrocytes, 01434	FUJIFILM Cellular Dynamics, Inc. (FCDI)	C1037
iCell GABANeurons Kit, 01279‡	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1011
• iCell GABANeurons, 01279	FUJIFILM Cellular Dynamics, Inc. (FCDI)	C1008
• iCell Neural Base Medium 1, 100 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1010
• iCell Neural Supplement A (50X), 2 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1032

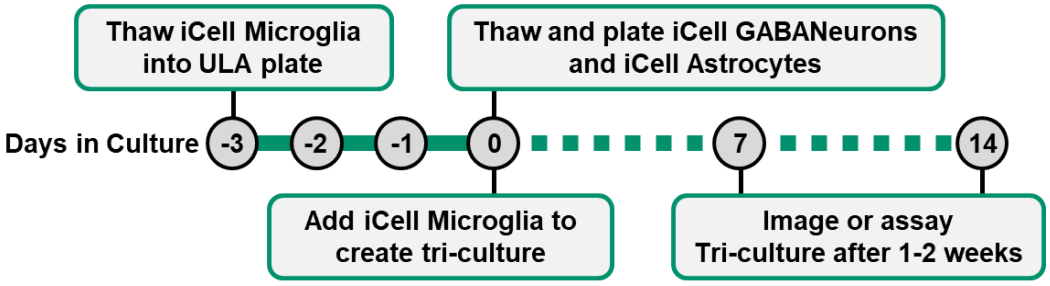
Item	Vendor	Catalog No.
Medium and Supplements ^		
iCell Microglia Supplement A (100X), 0.5 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	M1036
Plates and ECM		
6-well Clear, Flat Bottom, Ultra-Low Attachment (ULA) Plate	Corning	3471
96-well Cell Culture Microplate, µClear	Greiner Bio-One	655090
Polyethyleneimine (PEI) Solution, 50%	Sigma-Aldrich	181978
Borate Buffer (20X)	Thermo Fisher Scientific	28341
Geltrex™ Ready-to-Use, Basement Membrane Matrix	Thermo Fisher Scientific	A1569601

‡ This application protocol was developed primarily with iCell GABANeurons, 01279, however, additional testing was performed with iCell GABANeurons, 01434 (Cat. No. C1012) and iCell GlutaNeurons, 01279 (Cat. No. C1033). Culture conditions, including plating density, ECM, and media, were the same for all types of iPSC-derived neurons used.
^ iCell Microglia Supplement A (M1036) can be purchased separately

Workflow

A recommended experimental set-up is below. Cells can be assayed or imaged any time after 1-2 weeks in culture:

- Day (-3): Prepare Thawing Medium and Culturing Medium. Thaw iCell Microglia into a 6-well ULA plate.
- Day (-1): Coat 96-well plate with PEI.
- Day 0: Prepare Tri-Culture Medium. Thaw iCell GABANeurons and iCell Astrocytes. Harvest iCell Microglia. Plate cells in ratio of **6:1:1** Neurons:Astrocytes:Microglia in Tri-Culture Medium.
- Day 2-14: Replace 50% of the spent culture medium with Tri-Culture Medium every 2-3 days.
- Day 14 (or day of assay): Immunofluorescent staining, ELISA, phagocytosis, etc.



Tips Before Starting

1. Refer to the User's Guides for iCell GABANeurons and the Quick Guides for iCell Astrocytes and iCell Microglia for information on storage and handling of the cells.
   - iCell GABANeurons: https://www.fujifilmcdi.com/wp/wp-content/uploads/2020/07/CDI_iCellGABANeurons_UG.pdf
   - iCell Astrocytes: https://www.fujifilmcdi.com/wp/wp-content/uploads/2020/07/FCDI_iCellAstrocytes_QG.pdf
   - iCell Microglia: https://www.fujifilmcdi.com/wp/wp-content/uploads/2020/10/FCDI_iCellMicroglia_QG201029.pdf
2. Prepare an intermediate 10% PEI solution from the 50% stock to obtain more easily the final 0.1% working solution.

3. Addition of Penicillin-Streptomycin into Tri-Culture Medium is optional.
4. Store a bottle or an aliquot of DPBS (-/-) at 4°C as it will be needed during harvest of iCell Microglia.

Methods

Thawing iCell Microglia – Day (-3)

1. Prepare 25 ml of Thawing Medium according to the table below.

Thawing Medium – 25 ml

Component	Volume	Final Concentration
iCell Glial Base Medium	24 ml	N/A
iCell Neural Supplement C (25X)	1 ml	1X

2. Prepare 10 ml of Culturing Medium according to the table below.

Culturing Medium – 10 ml

Component	Volume	Final Concentration
iCell Glial Base Medium	9.4 ml	N/A
iCell Microglia Supplement A (100X)	0.1 ml	1X
iCell Microglia Supplement B (100X)	0.1 ml	1X
iCell Neural Supplement C (25X)	0.4 ml	1X

3. Thaw cells according to the iCell Microglia Quick Guide using Thawing Medium in place of Complete Maintenance Medium.
4. Resuspend the cells in 4-5 ml of Culturing Medium and distribute equally (counting the cells is not necessary) into 2 wells of a 6-well ULA plate.
5. Culture the cells in an incubator at 37ºC, 5% CO_2 for 3 days prior to initiation of tri-culture.

Preparing the PEI Solution – Day (-1)

Note: *Other sources of PEI have been tested with equivalent results, including Sigma-Aldrich Cat. No. P3143.*

1. Prepare an intermediate solution of PEI (10% w/w) by pouring 1-2 ml of the 50% PEI stock solution into a tared 15 ml centrifuge tube. Centrifuge at 400 × *g* for 5 minutes.
2. Weigh out the centrifuge tube to determine the weight (in grams) of PEI. The 50% PEI stock solution can be diluted with water to a known volume (density of water is 1.0 g/ml, thus 1 ml = 1 g).

   Note: *An example calculation is 2 g of the 50% PEI stock solution is diluted with 8 ml of sterile water to a final volume of 10 ml to obtain an intermediate 10% PEI solution (w/w).*
3. Mix the intermediate 10% PEI solution in the centrifuge tube to dissolve completely.

   Note: *PEI is a viscous material and may require extended vortexing or rocking overnight to dissolve.*
4. Prepare 50 ml of a working 0.1% PEI solution in 1X borate buffer by diluting 0.5 ml of the intermediate 10% PEI solution and 2.5 ml of 20X borate buffer with 47 ml of water.
5. Sterile filter the working 0.1% PEI solution through a disposable vacuum filter unit (e.g., Steriflip™ from Millipore Sigma Cat. No. SCGP00525).

   Note: *It is recommended to make the working 0.1% PEI solution fresh before each use, though it can be made a day in advance and stored at 4°C for up to 1 week.*

Coating the Plate with PEI – Day (-1) and Geltrex – Day 0

1. On Day (-1), the day before thawing neurons and astrocytes, add 80 µl/well of the working 0.1% PEI solution to a 96-well plate and incubate for 1 hour at 37°C.
2. Aspirate the PEI solution from the plate. Do not allow the wells to dry out.
3. Immediately, rinse the 96-well plate with ≥300 µl/well of sterile DPBS three times (3X) and finish with one rinse with ≥300 µl/well of sterile water.
4. Allow the 96-well plate to air-dry overnight in a sterile biological safety cabinet.

   Note: *It is critical to allow the plate to dry completely to achieve optimal cell attachment.*
5. On Day 0, add 50 µl/well of Geltrex solution to the PEI coated plate and incubate for 1 hour at 37°C.

Preparing the Tri-Culture Medium – Day 0

1. Prepare 90 ml of the Tri-Culture Medium according to the table below.

Tri-Culture Medium – 90 ml

Component	Volume	Final Concentration
iCell Neural Base Medium 1	88 ml	N/A
iCell Neural Supplement A (50X)	1.8 ml	1X
iCell Microglia Supplement A (100X) ǂ	1.8 ml	1X

ǂ An additional unit of iCell Microglia Supplement A (M1036) will be required as iCell Microglia Kit (Cat. No. R1204) only contains 1 vial.

Note: *The Tri-Culture Medium can be prepared on Day (-1) if needed and stored at 4°C for up to two weeks. Addition of Penicillin-streptomycin to the Tri-Culture Medium is optional.*

2. Allow the Tri-Culture Medium to equilibrate to room temperature for 15 minutes prior to use.

Harvesting iCell Microglia from ULA plates – Day 0

Note: *Chill a bottle of DPBS (-/-) at 4°C in advance as it will be needed during this step.*

1. Remove the ULA plate from the incubator containing the iCell Microglia plated 3 days prior.
2. Rinse iCell Microglia from each well of the ULA plate by drawing up existing media with a serological pipette, then tilt the plate toward you at a 45° angle and squirt the well from the top allowing the media to trickle down the surface (known as the “waterfall technique”). Repeat 4-5 times.
3. Transfer the cell suspension into a sterile 50 ml centrifuge tube.
4. Add 2 ml of cold DPBS to each well of the ULA plate and incubate at 4°C for 10 minutes.

   Note: *It is possible to begin thawing neurons and astrocytes in the next section during this incubation period (while waiting for the microglia to detach at 4°C).*
5. Check the cells under a microscope to make sure they have detached from the ULA plate.
6. Collect the DPBS cell suspension using the same waterfall technique and transfer to the 50 ml centrifuge tube.
7. Repeat the steps above until microglia are completely detached from the ULA plate.
8. Centrifuge the harvested iCell Microglia at 1000 x *g* for 10 minutes.
9. Aspirate the supernatant, resuspend the cells in 1 ml of Tri-Culture Medium, and remove an aliquot to count the cells using a hemocytometer.

Note: *The Certificate of Analysis (CoA) for iCell Microglia can be found online: fujifilmcdi.com/resources/coa-lookup/. Following culture on a ULA plate for 3 days, expect to recover ≥0.8 million viable microglia.*

10. Based on the numbers from the manual cell count above, resuspend iCell Microglia with Tri-Culture Medium to a density of 320,000 cells/ml.

Thawing iCell GABANeurons and iCell Astrocytes – Day 0

1. Thaw iCell GABANeurons according to the detailed instructions in the User's Guide using Thawing Medium in place of Complete Maintenance Medium.
2. Based on the number of viable cells listed on the CoA for iCell GABANeurons, resuspend the cell pellet following centrifugation with Tri-Culture Medium to a density of 1 million cells/ml. The CoA can be found online: fujifilmcdi.com/resources/coa-lookup/.

   Note: *It is recommended to perform a manual cell count using a hemocytometer to verify the cell viability and total number of viable cells listed in the CoA. Expect to recover ≥4 million viable neurons. Adjust cell density if necessary.*
3. Set the cell suspension of neurons aside and proceed to thawing of iCell Astrocytes.
4. Thaw iCell Astrocytes according to the detailed instructions in the Quick Guide using Thawing Medium in place of Maintenance Medium.
5. Based on the number listed on the Certificate of Testing (CoT) for iCell Astrocytes, resuspend the cell pellet following centrifugation with Tri-Culture Medium to a density of 320,000 cells/ml. The CoT can be found online using the same link above.

   Note: *It is recommended to perform a manual cell count using a hemocytometer to verify the cell viability and total number of viable cells listed in the CoT. Expect to recover ≥1 million viable astrocytes. Adjust cell density if necessary.*
6. Set the cell suspension of astrocytes aside and return to harvesting of iCell Microglia.

Preparing the Tri-Culture System – Day 0

Note: *It is recommended to mix each of the cell suspensions by inversion prior to plating and then dispense into a sterile reagent reservoir in order to plate cells using a multi-channel pipette.*

1. Manually aspirate the Geltrex solution from the 96-well plate after all three cell suspensions have been prepared.
2. Add 50 µl of the iCell GABANeurons cell suspension to the appropriate wells of the 96-well plate. Recommended cell seeding density for neurons is 50,000 cells/well.
3. Then add 25 µl of the iCell Astrocytes cell suspension to the appropriate wells of the 96-well plate. Recommended cell seeding density for astrocytes is 8,000 cells/well.
4. Finally, add 25 µl of the iCell Microglia cell suspension to the appropriate wells of the 96-well plate. Recommended cell seeding density for microglia is 8,000 cells/well.

   Note: *This approach enables comparison of mono-culture vs. tri-culture, for example. If any wells on the plate do not receive a particular cell type, make up the difference in volume with Tri-Culture Medium. Alternatively, all three cell types could be mixed and plated in a final volume of 100 µl if desired.*
5. Incubate the 96-well tri-culture plate at 37°C, 5% CO_2.

Maintaining the Tri-culture System – Day 2 and Beyond

1. Starting on Day 2 post-plating, replace 50% of the spent medium with fresh Tri-Culture Medium.
2. Continue to change the medium every 2-3 days and culture the cells at 37°C, 5% CO_2 until ready for imaging or assay.

Immunofluorescent Staining (optional)

Note: *This section provides basic guidance on how to stain iCell GABANeurons, iCell Astrocytes, and iCell Microglia in the tri-culture system for high content imaging. Volumes and incubation times are guidelines, not required.*

1. Fix the cells with 4% paraformaldehyde (PFA) solution at room temperature.

   Note: *First add an equal volume of 4% PFA to the cells without removing Tri-Culture Medium from the wells for ≥15 minutes. Then slowly aspirate this mixture and add 4% PFA to the cells again for ≥15 minutes.*

2. Remove PFA solution and add blocking buffer, which is composed of 10% FBS (v/v) and 0.1% Triton X-100 (v/v) in DPBS and incubate for ≥1 hour at room temperature.
3. Prepare a primary antibody mixture by diluting all three (3) antibodies together in blocking buffer according the table below.
4. Aspirate blocking buffer and add primary antibody mixture (in blocking buffer). Incubate for ≥2 hours at room temperature.
5. Carefully rinse the cells with DPBS three times.
6. Prepare a secondary antibody mixture by diluting all three (3) secondary antibodies together in blocking buffer according to the table below.
7. Aspirate DPBS wash and add secondary antibody mixture. Incubate for ≥1 hour at room temperature in the dark.
8. Carefully rinse the cells with DPBS three times.
9. Leave the cells in DPBS and proceed to viewing the plate on a preferred imaging system.

Reagents for Immunofluorescent Staining

Antibody	Vendor	Catalog No.	Host	Dilution
Milli-Mark™ Pan Neuronal Marker	Millipore	MAB2300	Mouse	1:1500
Anti-Iba1	FUJIFILM Wako Chemicals	019-19741	Rabbit	1:500
Anti-GFAP	Abcam	Ab4674	Chicken	1:500
Goat anti-Mouse IgG1 Secondary Antibody, Alexa Fluor 488	Invitrogen	A-21121	Goat	1:1000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568	Invitrogen	A-11011	Goat	1:1000
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 647	Invitrogen	A-21449	Goat	1:1000
Hoechst 33342 Solution (20 mM)	Thermo Fisher	66249	NA	1:10,000

Representative Data

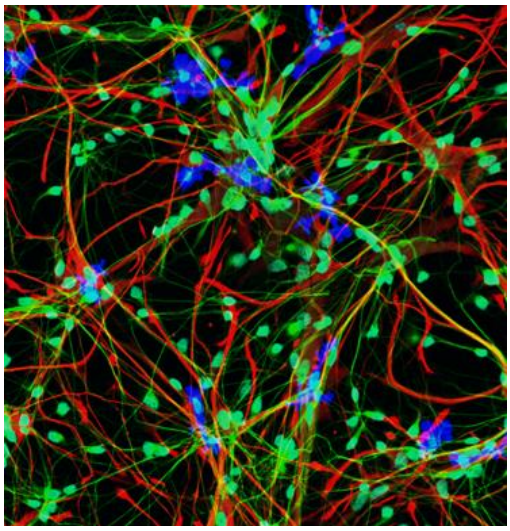


Figure 1. Imaging a human iPSC-derived "brain-in-a-dish". *This image shows iCell GABANeurons, iCell Astrocytes, and iCell Microglia (6:1:1 ratio) cultured in Tri-Culture Medium that was optimized for microglia survival. Cells were stained on Day 14 post-thaw using the following antibodies: pan neuronal marker (green, mouse), anti-GFAP (red, chicken), and anti-Iba1 from FUJIFILM Wako (blue, rabbit). High content imaging or confocal microscopy systems can be used to analyze cellular morphology and/or quantify cell numbers in the tri-culture system.*

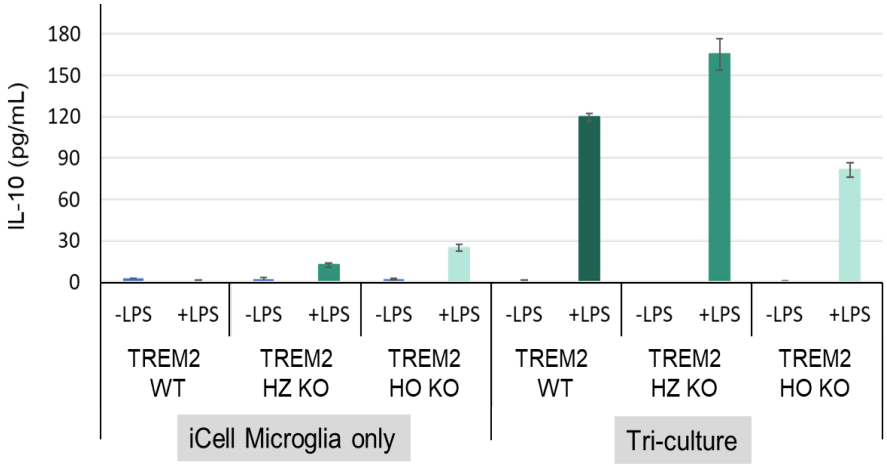


Figure 2. Investigating the role of TREM2 in neuroinflammation. *Tri-culture systems with iCell GABANeurons and iCell Astrocytes were prepared using iCell Microglia to study neuroinflammation. TREM2 "wild-type" (WT) and two different gene-edited TREM2 knockout (KO) [either heterozygous (HZ) or homozygous (HO)] iPSC lines were made into microglia and tested. On Day 14, cells were stimulated overnight ± LPS (1 µg/ml) and the cytokines released were quantified on the Luminex platform. Representative data selected for IL-10, showing the M2 "anti-inflammatory" pathway response in microglia, indicate that more IL-10 is released upon treatment of the tri-culture system than with microglia alone.*

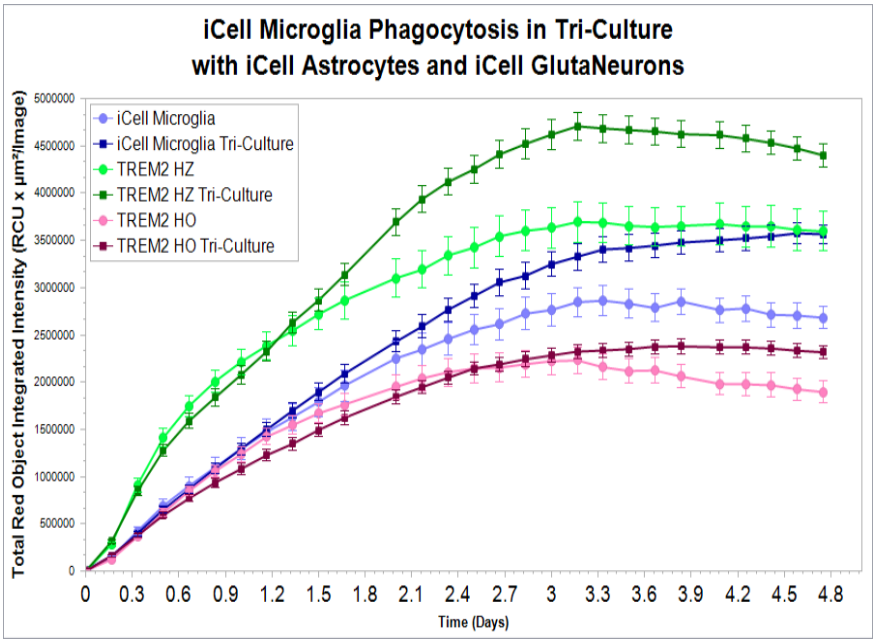


Figure 3. Interrogating the functional role of TREM2 in microglia phagocytosis. *Tri-culture systems were prepared with iCell GlutaNeurons and iCell Astrocytes using either apparently healthy normal (or WT) iCell Microglia (**blue**) and two different disease models derived from gene-edited iPSC lines, heterozygous (HZ; **green**) and homozygous (HO; **red**) TREM2 knockout (KO). Cells were plated together in 96-well plate format on Day 0 (including "control" wells for microglia in monoculture on the same plate) and on Day 1, pHrodo Red-labeled amyloid beta was added to the cells and phagocytosis was monitored in real-time via live-cell imaging on the Incucyte S3 system (Sartorius). The kinetic profile of microglia in tri-culture was, in general, improved over mono-culture alone.*

Summary

iCell Microglia play an important role in the study of neurodegeneration and neuroinflammation. Harnessing the power of iPSC technology to enable disease modeling through the use of gene-editing techniques or patient-derived lines further underscores the value of these cells. Combining other iCell products together with iCell Microglia to create a “brain-in-a-dish” tri-culture system, with a media and protocol that promote both survival and function of all three cell types, is a critical step forward towards developing human disease models with increased relevance that are more translatable to the clinic.

Revision History
Version 1.0: January
AP-MGL_TRI-CULTURE_20JAN22