

A versatile toolbox of human iPSC-derived microglia for disease modelling and multicellular *in vitro* models for neurodegeneration drug discovery

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1. Human ioMicroglia are ready to use after 4 days and mature after 10 days

A. ioMicroglia are shipped cryopreserved and are programmed to maturity upon revival. The protocol is in 3 phases. Phase 1: stabilisation for 24 hours with doxycycline. Phase 2: maturation for 9 days. Phase 3: maintenance. Cells are in optimal condition to use from day 10 post-thaw.

B. ioMicroglia maintain lot-to-lot consistency between production runs carried out by different operators. Comparing differentially expressed genes (DEGs) from bulk RNA-seq analysis of the three lots of ioMicroglia identified only 3 total DEGs, present between lots 2 and 3.

C. Bulk RNA-sequencing of three independent lots of male and female ioMicroglia at three different time points. Principal component analysis represents the variance in gene expression between the lots of ioMicroglia and shows high consistency across each lot at each given timepoint.

A) Phases of ioMicroglia development

B) ioMicroglia have high lot-to-lot consistency

C) PCA of ioMicroglia bulk RNA-seq throughout programming

2. ioMicroglia from male and female backgrounds display typical morphology and key functionalities

A. Brightfield images of ioMicroglia from female and male backgrounds displaying ramified morphology at day 10 post-thaw. 10x magnification.

B. Immunocytochemistry (ICC) on day 10 post-thaw with homogenous expression of microglia marker P2RY12 (green), with DAPI counterstain (blue). 10x magnification.

C. Day 10 female and male ioMicroglia were stimulated with LPS (100 ng/mL) and IFN- γ (20 ng/mL), or synthetic amyloid- β 1-42 oligomer (A β 42, 10 μ M, StressMarq) for 24 hours. ioMicroglia secrete IL-1 β , IL-6, IL-8 and TNF- α (as well as IL-12p70 and IL-10, data not shown).

D. Male donor-derived ioMicroglia 3-, 7- and 10-days post-thaw were stimulated with LPS (100 ng/mL) and IFN- γ (20 ng/mL) for 24 hours. Cytokine secretion was assessed by MSD Multiplex Assay. ioMicroglia secrete cytokines as early as 3 days post-thaw. Cytokine secretion levels are highest at day 10 post-thaw.

E. NanoLive Imaging showing phagocytosis of pHrodo™ RED labelled Zymosan particles by female ioMicroglia (top). Phagocytosis assay performed on day 10 male ioMicroglia using A β 42 AF488 (125 nM, Anaspec), pHrodo™ RED *E. coli* and pHrodo™ RED *S. aureus* beads. Representative images of phagocytosis post-incubation (bottom).

F. Quantitation of female and male ioMicroglia phagocytosis 4-, 6- and 10-days post-thaw. ioMicroglia were incubated with 1 μ g/0.33 cm² pHrodo™ RED labelled *E. coli* bioparticles for 24 hours. Images were acquired every hour on the Incucyte® S3 (Sartorius) identifying red fluorescence signal as a proportion of cells (left) and red fluorescence signal intensity per cell (right). Three technical replicates were performed per lot.

A) Brightfield

B) P2RY12

C) ioMicroglia secrete cytokines in response to LPS/IFN- γ and A β 42 oligomer

D) ioMicroglia secrete cytokines by day 3 post-thaw

E) Phagocytosis of female donor derived microglia

F) ioMicroglia robustly phagocytose bioparticles by day 4 post-thaw

3. ioMicroglia Alzheimer's disease models demonstrate relevant disease-related phenotypes

Disease models

Gene	Zygosity	Mutation
APOE	Het	C112R/WT (4/3)
	Hom	C112R/C112R (4/4)
TREM2	Het	R47H/WT
	Hom	R47H/R47H

ioMicroglia Alzheimer's disease models with AD mutations in APOE and TREM2.

A. TREM2 disease model phagocytosis. Day 10 ioMicroglia were incubated with 1 μ g/0.33 cm² pHrodo™ RED labelled *E. coli* particles, or AF488 labelled A β 42 (Anaspec) for 24 hours. Percentage of cells phagocytosing *E. coli* particles over 24 hours (left) and red fluorescence signal intensity per cell (right). Images acquired every hour on the Incucyte® S3 (Sartorius). Three technical replicates per experiment.

B. As above for the APOE disease model ioMicroglia.

C. Cytokine response after stimulation with LPS (100 ng/mL) and IFN- γ (20 ng/mL), or synthetic A β 42 oligomer (10 μ M, StressMarq) at day 10 post-thaw. Cytokine secretion assessed by MSD Multiplex Assay. Secretion observed for IL-6, IL-8 and IL-1 β (as well as IFN- γ , TNF- α , IL-4, IL-10, IL-12p70, data not shown).

A) TREM2 disease model (R47H) phagocytosis

B) APOE disease model (C112R) phagocytosis

C) APOE disease model (C112R) cytokine release

4. Ablation of ATP-mediated chemotaxis in ioMicroglia P2RY12 knockout models

Clear ablation of ATP-mediated chemotaxis is seen in ioMicroglia P2RY12 knockout models

ioMicroglia Male (WT), ioMicroglia P2RY12 null/null, and ioMicroglia P2RY12 null/WT were plated onto Incucyte® Clearview 96-well Plate for Chemotaxis with 8 μ m sized pores and 25 μ M ATP in the basal compartment.

Cell movement through the pores onto the underside of the membrane was quantified with the Incucyte® S3 Live-Cell Analysis System using the Incucyte® Chemotaxis Analysis software (in collaboration with Sartorius).

A. The ratio of membrane coverage of the underside of the membrane to the top is plotted. N=6, two independent experiments, mean and SEM are shown

B. Representative brightfield imaging of the underside of the transwell membranes at 62 h, blue shows the applied cell masking on each image.

These data were collected in collaboration with Sartorius.

A) Ratio of cell numbers in bottom vs top transwell membrane

B) Underside of transwell membranes after 62 h

5. ioMicroglia are a more physiologically relevant model of human microglia than HMC3 cells

A. PCA of bulk RNA-sequencing data from day 10 male and female ioMicroglia and the immortalised HMC3 cell line. Data has been integrated with information reported by Abud *et al.* 2018 (1).

Bulk RNA-sequencing data shows that ioMicroglia cluster closely to primary foetal and adult microglia data sets derived from this publication, whereas the HMC3s cluster closely with iPSCs.

B. Secretion of cytokines in response to 24 h stimulation with LPS (100 ng/mL) and IFN- γ (20 ng/mL) by ioMicroglia and HMC3. Cytokine secretion assessed by MSD Multiplex Assay. Quantitation of IL-1 β (left), IL-6 (middle) and TNF- α (right). HMC3 cytokine secretion is 1000x to 10x less than ioMicroglia.

A) PCA comparing ioMicroglia, HMC3, and primary cells

B) Cytokine secretion in response to immunostimulation

6. ioMicroglia in multi-cell cultures with ioAstrocytes and ioGlutamatergic Neurons

A. Optimised tri-culture schematic. Astrocytes were cultured to day 4 before addition of ioGlutamatergic Neurons. Separately, ioMicroglia were cultured to day 10, before being reseeded onto the day 14 co-culture. The tri-culture was maintained for a further 7 days.

B. High-resolution ICC analysis confirms the successful co-localisation and morphological health of three distinct cell types within a unified environment. By day 7, the protocol yields a highly consistent, integrated network suitable for complex cell modelling. DAPI (blue) highlights the total cell density and integrity of the culture.

C. ioGlutamatergic Neurons expressing Incucyte® Neuroburst Orange Lentivirus. ioGlutamatergic Neurons were cultured with and without ioMicroglia. Calcium burst intensity, measured by orange calcium units (OCU), was recorded using the Incucyte® Neuronal Activity Analysis software. Representative calcium traces shown for each culture condition at 15 days post-microglia addition (left). Quantification of network correlation and mean burst duration after 15 days of co-culture. Data presented as mean $\pm$ SEM, n = 3, 12 replicates (right). ioGN = ioGlutamatergic Neurons only, ioGN+ioMG = ioGlutamatergic Neurons co-cultured with ioMicroglia. Data generated by Jasmine Trigg and colleagues at Sartorius, Cambridge UK.

A) Tri-culture protocol schematic

B) Tri-culture ICC analysis

C) ioMicroglia enhance neural network activity in co-culture

7. Functional 3D bioprinting and organoid neuroinflammation models with ioMicroglia

A. ioMicroglia integrate in BrainZell organoids shown by IBA1 staining detected within the organoid structure (left). IBA1 gene expression (qPCR) is proportional to the number of ioMicroglia added (data not shown). ioMicroglia retain functionality within the organoid, demonstrated by proportionally increasing levels of IL-6 secretion post LPS-stimulation which is reduced by dexamethasone treatment (right). Similar results for TNFa (data not shown). Data generated by Robin Pronk and colleagues at BrainZell.

B. Successful integration of these reporter microglia into a 3D bioprinting system, the Rastrum Allegro, and demonstrate a functional neuroinflammatory response to LPS, Amyloid β 1-42 and Nigericin treatment when in tri-culture with hiPSC-derived astrocytes and neurons. Data generated by Emma Jones and colleagues at Medicine Discovery Catapult.

A) ioMicroglia functionally integrate into brain organoids

B) Generation of 3D tri-cultures with GFP ioMicroglia

Summary & conclusions

opti-ox™-mediated deterministically programmed iPSC-derived microglia from diverse genetic backgrounds serve as a versatile platform for disease modelling and advanced co-culture systems.

Wild-type microglia display key characteristics and functionalities as early as 4 days post-revival, enabling the study of complex neuroinflammatory and neurodegenerative diseases.

A panel of disease model cells carrying Alzheimer's disease-relevant mutations including APOE^{C112R/C112R} and TREM2^{R47H/WT} are relevant for drug discovery research.

Wild-type microglia from male and female backgrounds consistently outperform the immortalised microglia-like HMC3 line, showing increased phagocytic capacity, upregulation of key microglia genes, and up to 1000-fold higher secretion of key cytokines following stimulation.

ioMicroglia form tri-cultures and co-cultures, supported by a fully optimised protocol developed at bit.bio.

3D organoid and bioprinting models showcase how ioMicroglia can be easily integrated to add a functional neuroinflammatory aspect.

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