A perfused three-dimensional culture model of human cortical tissue

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INTRODUCTION

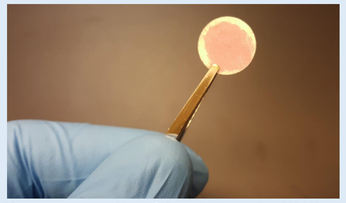
Human cortical tissue can be exceedingly difficult to model in vitro. Issues of cellular survival and maintaining in vivo-like function pervade, often affecting study validity and translatability. Planar cultures have long been the standard method of growing cells in vitro, but they fail to capture the complexities of the in vivo environment. This is particularly applicable to the delicate balance of microglia-mediated inflammatory responses. Three-dimensional (3D) cultures have been shown to be more physiologically relevant models of in vivo tissue. However, even they fail to fully mimic living tissue and suffer from the lack of active oxygen and nutrient transport. Lena Biosciences has developed a groundbreaking in vitro system that facilitates long-term survival of 3D cultures and significantly improves cellular metabolic function. PerfusionPal offers a simple method for simultaneously perfusing twelve statistically independent 3D cultures using only a single tube and pump. Utilizing a unique blood substitute, cultures grown in SeedEZ 3D scaffolds receive superior oxygenation, resulting in significant functional improvements and allowing for growth of denser cultures with increased longevity. Using this system, Lena Biosciences has developed a human cortical model comprising iPSC-derived neurons (acquired from BrainXell), primary astrocytes, and cells from the HMC3 microglia cell line. Both glutamatergic and GABAergic neurons were combined to generate a more accurate model. PerfusionPal enables the study of the complex interplay between pro- and anti-inflammatory responses to insult and the long-term effects of activating these pathways.

METHODS

Cell culture

HepG2 cells and HMC3 cells (ATCC) were maintained in EMEM supplemented with 10% fetal bovine serum in a humidified incubator at 37 ºC. Rat cortical cell cultures were generated by combining cells from two cortical tissue dissociations (BrainBits®, postnatal day 0 and embryonic day 18) and were cultured in Neurobasal medium supplemented with B-27 Supplement and GlutaMAX. Human cortical cultures comprising iPSC-derived neurons (Cellular Dynamics, BrainXell) and primary astrocytes (ScienCell), and HMC3 microglia cells were plated and maintained in iPSC neuronal medium or BrainXell's Cortical Neuron Complete Medium. Jurkat T cells (kindly provided by Nate Dwarshuis from the Roy lab) were maintained according to ATCC specifications. Primary human T cells (Astarte) were cultured and activated according to protocols from the Roy lab.

3D cultures were generated using Lena Biosciences' patented SeedEZ™ scaffolds:



HepG2 cells were seeded in Matrigel® (8 mg/mL) at a density of 300,000 cells per scaffold. Cultures were allowed to gel at 37 ºC for 15 minutes and subsequently transferred to Lena Biosciences' PerfusionPal system or to a multiwell plate ("Control"). Cultures in PerfusionPal were either maintained without perfusion ("Static") or subjected to perfusion at a rate of 16 culture volume changes per 24 hour period ("Perfused") for the duration of the study. 2D control cultures consisted of the same number of cells plated in 24-well plates.

Human cortical cultures were plated in medium or in Matrigel® (8 mg/mL) into SeedEZ™ scaffolds that had been coated with poly-D-lysine (PDL) (100 µg/mL) followed with MaxGel™ human extracellular matrix (ECM) (1 mg/mL) (both reagents from Sigma-Aldrich) or just PDL, respectively. The total cell density was 500,000 or1,000,000 cells/scaffold with an overall cellular ratio of 2:2:1 neurons:astrocytes:microglia. Perfusion conditions were the same as for HepG2 cells.

alamarBlue Assay for Metabolic Function

After the specified number of days *in vitro* (DIV), alamarBlue reagent was added to cultures at a volume equal to 1/10 of the volume of culture medium. The cultures were returned to the incubator for a period of 30 minutes to 2 hours (depending on cell type) after which a sample of the medium from each condition was transferred to a 96-well plate and read on a Biotek Synergy 4 plate reader for fluorescence (ex/em 545/590 nm). Data (HepG2) were normalized to cell number as determined by total LDH of a set of cell density standards described below.

LDH Assay for Total and Released LDH

Prior to the addition of alamarBlue reagent, a sample of medium was taken to assess LDH release. After the alamarBlue assay, the cultures were lysed with Triton X-100 (1%) for 1 hour at 37 ºC for determination of total LDH. In addition, a freshly plated set of HepG2 3D cultures with densities ranging from 200,000 – 4,000,000 cells/scaffold were lysed and used to generate a standard curve to determine cell number in each culture. A sample of medium from each condition was transferred to a 96-well plate for a colorimetric LDH assay. LDH release data were normalized to cell number.

Immunocytochemistry

The 3-D cultures were fixed with 2% paraformaldehyde for 30 minutes at room temperature. Whole cultures were incubated with primary antibody microtubule-associated protein (MAP2) and glial fibrillary acidic protein (GFAP) from Chemicon overnight at 4°C. Cultures were incubated with the appropriate secondary antibody (Jackson ImmunoResearch) for 1 hour at room temperature. Cellular nuclei were labeled using DAPI stain (Molecular Probes). Imaging was carried out using Neurolucida software controlling a Nikon Eclipse 80i upright microscope equipped with an Optronics MicroFIRE camera.

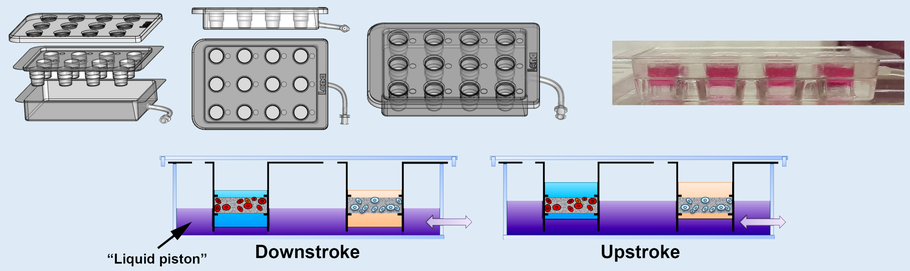
Cell Viability Assessment

After the specified number of DIV, cultures were removed from the PerfusionPal system and were exposed to Calcein AM (in DPBS) for a period of 30 minutes at room temperature followed by multiple rinses with DPBS. Imaging was carried out using Neurolucida software controlling a Nikon Eclipse 80i upright microscope equipped with an Optronics MicroFIRE camera.

Statistical Analysis

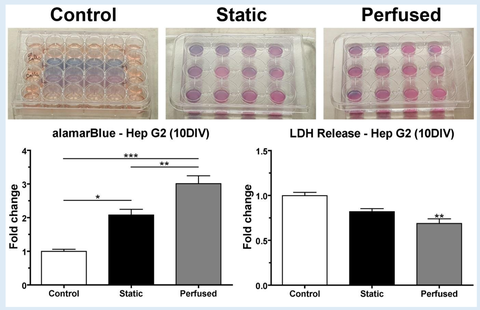
Statistical significance was determined by One-way ANOVA and Tukey's post-hoc test or by Two-way ANOVA.

LENABIOSCIENCES PerfusionPal System



Cells seeded in 3D hydrogels supported by a rigid scaffold were maintained in statistically independent wells in their own medium which floats atop a high-density blood-substitute in which medium is immiscible. The blood-substitute readily dissolves atmospheric gases at levels which are orders of magnitude higher than for aqueous medium. Infusion and withdrawal of this blood-substitute acts as a "liquid piston" and introduces perfusion into the system without the need for separate pumps and tubing for each culture condition.

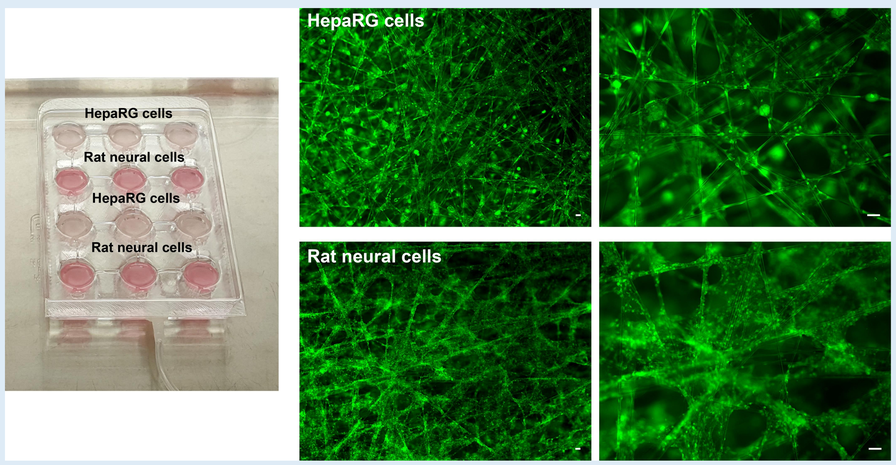
Cultures grown in PerfusionPal have increased metabolic function (respiration)



Culture of HepG2 cells in the PerfusionPal system consistently resulted in increased metabolic activity as measured by alamarBlue assay. HepG2 cells maintained in a 12-well system over a 10-day period showed a two-fold improvement in metabolic activity over cultures maintained in a standard multiwell plate when the blood-substitute was introduced ($p<0.05$), and a three-fold improvement when the cultures were perfused ($p<0.001$). Perfused cultures showed 50% more alamarBlue signal than static cultures ($p<0.01$).

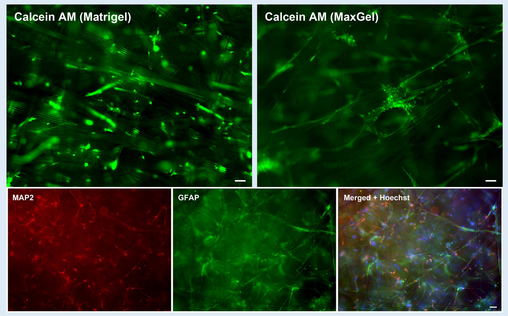
LDH release was also significantly decreased in perfused cultures ($p<0.01$) compared with control cultures, indicating improved cell viability presumably due to increased oxygenation.

PerfusionPal supports simultaneous, long-term culture of a rat cortical model and a human liver model

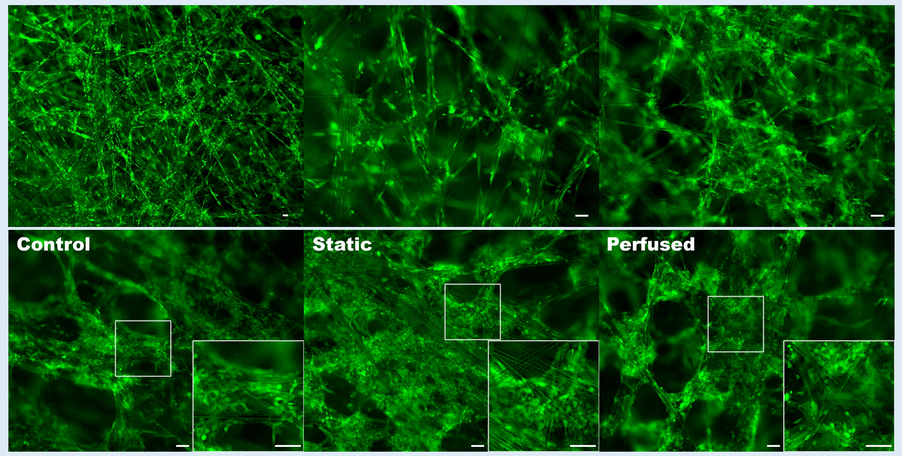


Multiple cell types can be maintained simultaneously in a single PerfusionPal system for a 4 weeks. Primary rat cortical cultures consisting of prenatally-derived neurons and postnatally-derived glial cells were cultured alongside HepaRG™ in a PerfusionPal system (6-wells each) for 28 days. Calcein AM images from this time show dense clusters of healthy cells at the nodes where fibers of the SeedEZ scaffold meet. The open structure of the scaffold permits perfused medium to flow between these clusters to deliver oxygen and nutrients more efficiently than can be achieved with diffusion alone. Scale bars: 50µm

Cell source and matrix optimization for human cortical cultures

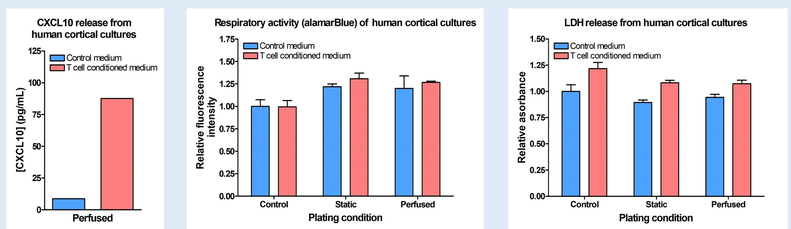


Human cortical cultures comprising iPSC-derived neurons and astrocytes (top row) as well as a microglia cell line (bottom row) show modest viability and weakly label for cellular markers. iCell® neurons and astrocytes combined at a 2:1 ratio in Matrigel® or in a SeedEZ scaffold coated with MaxGel™ (Sigma-Aldrich) showed reasonable viability after 5 days in culture as determined using Calcein AM (top row). Cell density and attachment were notably low, however. Neurons, astrocytes, and microglia combined at a 4:1:2 ratio in Matrigel® labeled with markers for neurons (MAP2) and astrocytes (GFAP) after 3 days in culture. Again, cell density and attachment were low. Scale bars: 50µm



Human cortical cultures comprising iPSC-derived neurons (BrainXell) and astrocytes as well as a microglia cell line are viable. iPSC derived neurons (Mixed Glutamatergic and GABAergic, BrainXell), astrocytes (ScienCell), and HMC3 microglia were combined at a 2:2:1 ratio in SeedEZ™ scaffolds coated with PDL followed by MaxGel™ human ECM. Cultures were maintained in BrainXell complete cortical neuron medium and showed high viability after 4 days in culture as determined using Calcein AM (top row). Cultures seeded into PDL-coated SeedEZ scaffolds with Matrigel® were maintained in BrainXell complete cortical medium and showed very high viability after 15 days in culture as determined using Calcein AM(bottom row). The cells formed dense, 3D structures that expanded well beyond the nucleation sites where fibers crossed. Scaffolds were placed into a multi-well plate ("Control"), an unperfused PerfusionPal system ("Static"), or a perfused PerfusionPal system ("Perfused"). Scale bars: 50µm

Effect of Pro-inflammatory stimuli on human cortical cultures



Human cortical cultures comprising iPSC-derived neurons (BrainXell) and astrocytes and HMC3 microglia release CXCL10 in response to conditioned medium from activated T cells. A pilot experiment showed that CXCL10 release from perfused cultures was increased in response to conditioned medium from human T cells (13 days in culture; 24 hour treatment). Conversion of alamarBlue when cultures were maintained in PerfusionPal without or with perfusion indicates a trend toward increased cellular respiration. LDH release from cortical cultures shows a decreasing trend when maintained in PerfusionPal. Plating condition was determined to be significant by Two-way ANOVA for both alamarBlue and LDH release.

CONCLUSIONS/FUTURE DIRECTIONS

- We have created a 3D model of human cortical tissue that shows high viability and tissue-like structure after two weeks *in vitro*.
- We have shown that the cultures are capable to responding to pro-inflammatory stimuli.
- The data suggest that the addition of blood substitute and perfusion may produce cultures with superior respiration and higher viability, though this will need to be substantiated with more rigorous assays and longer studies.
- The individual cellular components of the model will be characterized.
- The inflammatory response will be further characterized by measuring the release of other cytokines chemokines.

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