Modeling 3D Liver Tissue:

3D Hepatocyte Spheroids in Low Attachment Plates

Introduction

A growing body of literature suggests that moving from traditional 2D culture to 3D formats yields a more liver-like environment for hepatocyte assays and generates more predictive biology in vitro. iCell® Hepatocytes 2.0, human induced pluripotent stem cell-derived hepatocytes, exhibit typical hepatic functionality and phenotypic stability. Due to their human origin, native cell-like behavior, and ease of use, iCell Hepatocytes 2.0 represent an optimal test system for basic hepatic biology in all areas of drug development, disease modeling, and toxicology.

This Application Protocol presents a workflow for forming liver spheroids with iCell Hepatocytes 2.0 in ultra-low attachment plates that enables the researcher to control the size of the engineered tissue.

Required Consumables

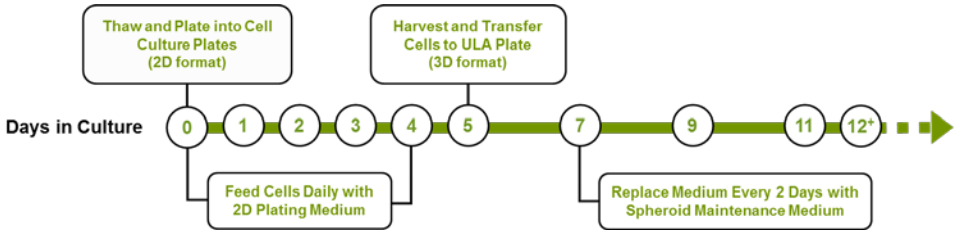
The following consumables are required in addition to the materials specified in the iCell Hepatocytes 2.0 User's Guide.

Item	Vendor	Catalog Number
iCell Hepatocytes 2.0 Kit (Hepatocytes)	Cellular Dynamics International (CDI)	PHC-100-020-001
DMEM/F-12 Medium, No Phenol Red	Thermo Fisher Scientific	11039-021
B-27 Supplement, 50X	Thermo Fisher Scientific	17504-044
Dexamethasone	Fisher Scientific	ICN19456125
Gentamicin, 50 mg/ml	Thermo Fisher Scientific	15750-060
Oncostatin M, 10 µg/ml	R&D Systems	295-OM
BioCoat Collagen I Multiwell Plate (Cell Culture Plate)	Corning	354400 (6-well) 354500 (12-well) 354408 (24-well) 354505 (48-well) 354407 (96-well)
InSphero GravityTRAP ULA 96-well Plate (ULA Plate)*	PerkinElmer	ISP-09-001
Sterile Reservoirs, 25 - 100 ml	Multiple Vendors	
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Thermo Fisher Scientific	14190-144
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher Scientific	A11105-01
Geltrex hESC-qualified Ready-To-Use Reduced Growth Factor Basement Membrane Matrix (Geltrex)	Thermo Fisher Scientific	A15696-01

* The 3D spheroid formation procedures described here were optimized using either the InSphero GravityTRAP ULA 96-well Plate (PerkinElmer, Cat. No. ISP-09-001) or Corning ULA Spheroid 96-well or 384-well Microplates (Corning, Cat. No. 4515 or 4516); ULA = ultra-low attachment

Workflow

iCell Hepatocytes 2.0 are thawed and plated into a collagen I-coated multiwell cell culture plate. On days 1 through 4 post-plating, spent medium is replaced daily. iCell Hepatocytes 2.0 can be harvested between days 5 and 9 post-plating for preparing 3D spheroid cultures. The cells are transferred to a ULA plate and a portion of the medium is replaced every 2 days thereafter. The cells form spheroids within 48 hours after transfer to the ULA plate and can be maintained for at least 35 days.



Methods

Culturing Hepatocytes

Thaw and maintain iCell Hepatocytes 2.0 according to their User's Guide until ready to form 3D spheroids. In place of the Plating Medium and Maintenance Medium recommended in the User's Guide, prepare and use the 2D-Plating Medium and Spheroid Maintenance Medium described below.

Note: *Dexamethasone is a known inducer of cytochrome P450 3A4 (CYP3A4). When testing the induction of CYP3A4 by compound treatment, do not include dexamethasone in the Spheroid Maintenance Medium to lower CYP3A4 basal activity. For optimal long-term health, however, spheroids should not be maintained without dexamethasone for longer than 5 - 7 days.*

2D-Plating Medium[1,2,3,4]		
Component	**Amount (ml)**	**Final Concentration**
DMEM/F-12 Medium, No Phenol Red	98	98%
B27 Supplement, 50X	2	1X
Dexamethasone, 5 mM	0.002	0.1 µM
Gentamicin, 50 mg/ml	0.05	25 µg/ml
Oncostatin M, 10 µg/ml	0.2	20 ng/ml

1 Prepare the 2D-Plating Medium fresh on the day that the cells are thawed to ensure optimal viability of cells upon plating.

2 Follow the manufacturer's instructions for storage and reconstitution of component stock solutions.

3 Filter the medium using a 150 ml, 0.2 µm PES filter unit.

4 Store the 2D-Plating Medium at 4°C for up to 1 week. Do not store at -20°C.

Notes

Notes

Spheroid Maintenance Medium[1,2,3,4]		
Component	**Amount (ml)**	**Final Concentration**
DMEM/F-12 Medium	98	98%
B27 Supplement, 50X	2	1X
Dexamethasone, 5 mM	0.002	0.1 µM
Gentamicin, 50 mg/ml	0.05	25 µg/ml

1 Prepare the Spheroid Maintenance Medium on day 5.

2 Follow the manufacturer's instructions for storage and reconstitution of component stock solutions.

3 Filter the medium using a 150 ml, 0.2 µm PES filter unit.

4 Store the Spheroid Maintenance Medium at 4°C for up to 1 week. Do not store at -20°C.

Harvesting Hepatocytes

iCell Hepatocytes 2.0 can be harvested from a 2D cell culture plate between days 5 and 9 post-plating for preparing 3D spheroid cultures. Appropriately harvesting hepatocytes from the cell culture plate(s) as described here is a critical step to achieve robust functionality upon transfer to the ULA plate for 3D spheroid formation. The following recommendations detail proper handling procedures and volumes of reagents per well of a multiwell cell culture plate. Scale volumes appropriately for other formats.

Cell Culture Vessel (2D format)	Cell Number Expected to be Recovered	Volume of D-PBS Wash (ml)	Volume of Accutase (ml)	Volume of Spheroid Maint. Medium Rinse (ml)
6-well Cell Culture Plate	~1,000,000	2	1	2
12-well Cell Culture Plate	~400,000	1	0.5	1
24-well Cell Culture Plate	~200,000	0.6	0.3	0.6
48-well Cell Culture Plate	~100,000	0.3	0.15	0.3
96-well Cell Culture Plate	~30,000	0.1	0.05	0.1

Table 1: Summary of Recommended Volumes and Measures for Dissociation
All volumes and measures are per well.

1. Before use, equilibrate an aliquot of Spheroid Maintenance Medium and D-PBS to room temperature.
2. Remove the spent medium from the cell culture plate containing the hepatocytes.
3. Wash the hepatocytes once with a volume of D-PBS (Table 1).
4. Add a volume of Accutase to the cells (Table 1).

   Note: *Do not use trypsin to dissociate the hepatocytes.*
5. Monitor dissociation with a microscope and incubate the cell culture plate at room temperature until the cells just begin to detach from the plate (~2 - 4 minutes, Figure 1B).

6. Add a rinse volume of Spheroid Maintenance Medium to dilute the Accutase (Table 1).
7. Gently pipette up and down several times around the well to ensure the cells are detached and to generate a cell suspension.

   Note: *The cell suspension contains mostly small clusters of cells. Do not attempt to singularize the cells. Over-digestion of the hepatocytes impairs their functionality in the 3D spheroid culture.*

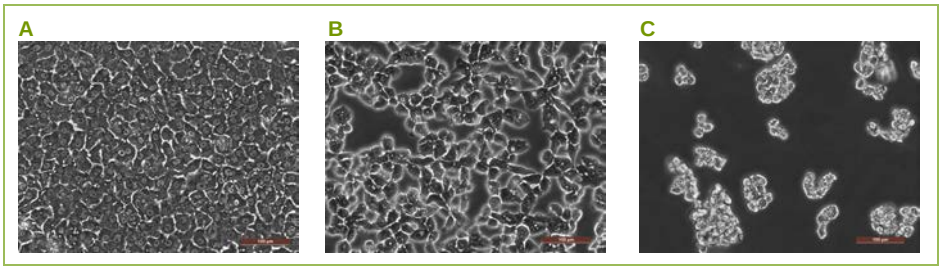


Figure 1: Hepatocytes before and after Enzymatic Treatment Using the Dissociation Reagent
iCell Hepatocytes 2.0 were cultured for 7 days in a 6-well cell culture plate: (A) before treatment, (B) after treatment, and (C) after being washed from the well as described in step 7.

8. Transfer the dissociated cells to a 15 ml centrifuge tube.
9. Add a rinse volume of Spheroid Maintenance Medium (Table 1). Transfer the medium rinse to the 15 ml centrifuge tube containing the cells.

   Note: *Dissociated cells from multiple wells can be pooled in the same centrifuge tube*.
10. Centrifuge the cell suspension at room temperature at 200 x g (or ~1,000 rpm) for 3 minutes.
11. Carefully aspirate the supernatant, taking care not to disturb the cell pellet.
12. Gently resuspend the cell pellet in the desired volume of Spheroid Maintenance Medium depending on the 2D culture format that was used and the desired target spheroid size (Table 2).

    CDI recommends preparing spheroids with diameters of ~150 - 300 microns (~1,000 - 2,000 cells) for common applications, such as hepatotoxicity assays or cytochrome P450 induction testing.

    Note: *Because the cell suspension contains mostly small clusters of cells, it is not amenable to counting. The cell number values listed in Table 2 are estimates based on the expected recovery numbers provided in Table 1.*

Notes

Cell Culture Vessel	Volume of Spheroid Maintenance Medium (ml)					
2D Format	**100 - 200 µm Diameter / ~500 Cells**	**150 - 250 µm Diameter / ~1,000 Cells**	**200 - 300 µm Diameter / ~2,000 Cells**	**250 - 350 µm Diameter / ~3,000 Cells**	**350 - 450 µm Diameter / ~5,000 Cells**	**450 - 550 µm Diameter / ~10,000 Cells**
6-well Cell Culture Plate	70	35	17.5	11.67	7	3.5
12-well Cell Culture Plate	28	14	7	4.67	2.8	1.4
24-well Cell Culture Plate	14	7	3.5	2.33	1.4	0.7
48-well Cell Culture Plate	7	3.5	1.75	1.17	0.7	0.35
96-well Cell Culture Plate	2.1	1.05	0.525	0.35	0.21	0.105

Table 2: Recommended Resuspension Volumes for Cells Harvested from 2D Culture
All volumes are per harvested well.

Seeding Hepatocytes for 3D Spheroid Formation

The following procedure details seeding the hepatocytes in the ULA plate for 3D spheroid formation.

1. Prepare a 20% (v/v) solution of Geltrex in Spheroid Maintenance Medium in a sterile reservoir. Mix well by gently rocking the reservoir several times.
2. Prepare the spheroid formation mixture by combining an equal volume of cell suspension with an equal volume of 20% solution of Geltrex in a sterile reservoir. Mix well by gently rocking the reservoir several times.
3. Dispense 70 µl/well of spheroid formation mixture to the ULA plate. Rock the reservoir periodically to ensure that the cells remain in suspension during the entire dispensing procedure.

   Note: *If the spheroid culture will be maintained for an extended period of time (e.g. more than 5 days), CDI recommends seeding only the inner wells of the ULA plate to avoid edge effects due to evaporation. Add the Spheroid Maintenance Medium or D-PBS to all outer wells and any wells not containing cells to minimize the occurrence of edge effects.*
4. Centrifuge the ULA plate at room temperature at 200 x g (or ~1,000 rpm) for 2 minutes to remove any air bubbles and settle the cells to the bottom of the wells.

5. Culture the ULA plate in a cell culture incubator at 37°C, 5% CO_2 for approximately 48 hours to allow spheroid formation to occur.

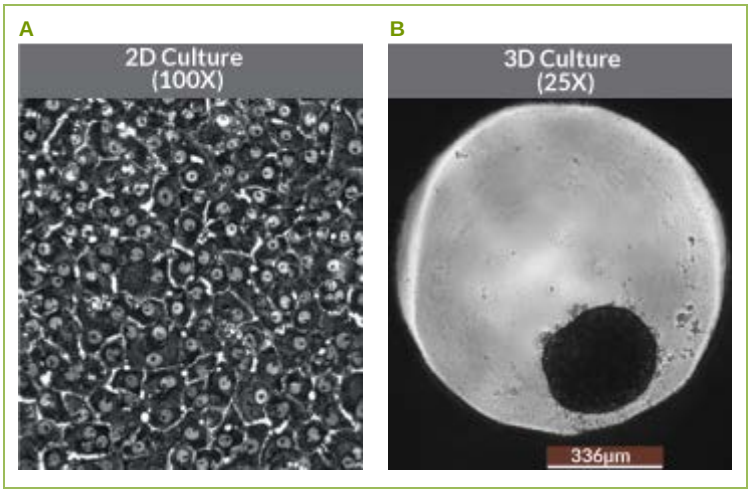


Figure 2: Phase Images of 2D and 3D Cultures of iCell Hepatocytes 2.0
(A) Cells are thawed and plated into the collagen-coated cell culture plate to form a 2D confluent monolayer. (B) Cells are then detached and seeded in the ULA plate to form 3D spheroids within 2 days.

Maintaining 3D Spheroids

Continue to culture the 3D spheroids in the ULA plate until the day of the desired endpoint assay. Starting day 2 post-plating and every 2 days thereafter, replace with fresh Spheroid Maintenance Medium.

1. Immediately before use, equilibrate an aliquot of Spheroid Maintenance Medium to room temperature.
2. Carefully remove 50 µl/well of spent medium:
   a. Place the pipette tips at an angle along the side of the wells. Do not touch the well's bottom.
   b. Aspirate at low pipetting speed (<30 µl/sec). A volume of ~20 µl will remain in the well's bottom.
3. Slowly add 50 µl/well of Spheroid Maintenance Medium.
4. Centrifuge the ULA plate at room temperature at 200 x g (or ~1,000 rpm) for 2 minutes.
5. Return the ULA plate to a cell culture incubator at 37°C, 5% CO_2.

Notes

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Summary

iCell Hepatocytes 2.0 can be cultured as 3D spheroid microtissues in ULA plates. Moving beyond traditional 2D cultures to 3D formats yields a more liver-like environment for hepatocyte assays and generates more predictive biology in vitro. The combination of iPSC technology with advanced culture techniques offers advantages over existing models via: (1) a uniform cell source in the form of iPSC-derived hepatocytes, thus removing the inter-lot and inter-donor variability observed with primary hepatocytes and (2) enhanced functional maturity of the cells imparted by the increased complexity of the culture conditions, thereby leading to enhanced predictive power. CDI is functionally characterizing the iCell Hepatocyte spheroids, observing that they exhibit prolonged survival beyond the already impressive 21 - 28 day lifespan observed in 2D culture on collagen-coated vessels. In addition to enhanced culturability, preliminary data suggest that 3D spheroid cultures demonstrate increased functional maturity.

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