HEPG2/C3A and HMEC1 co-cultured spheroids with forced aggregation
Protocol

This protocol describes the procedure for co-culturing of an immortalized hepatocyte cell line HEPG2/C3A and HMEC1 an immortalized human microvascular endothelial cell line in low adhesion microwell plates.

Reagents and Materials

- Equilibrated ClinoReactor®
- Cell counter
- Non-essential amino acids (NEAA)
- Foetal calf serum (FCS) or Foetal bovine serum (FBS)
- Hanks´Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}
- Penicillin/streptomycin 10 000 U /mL
- 10 ng/mL epidermal growth factor (EFG) (e.g. Sigma-Aldrich, E9644))
- 1 µg/mL hydrocortisone (Sigma-Aldrich, H2270).
- DMEM (low glucose, with pyruvate, no glutamine, no phenol red)
- Dimethyl sulfoxide (DMSO), sterile filtered
- 70% Ethanol solution
- Stable glutamine supplement (e.g., GlutaMax™)
- 0.4% Trypan Blue solution
- Trypsin-EDTA (0.5%)
- Isopropanol
- AggreWell™400 (Stemcell Technologies, 27845) and rinsing solution (Stemcell Technologies,
- Complete DMEM media (supplemented with 1% NEAA, 10% FCS, 0.5% Penicillin/streptomycin, 1% stable glutamine solution)
- Complete MCDB131 media (supplemented with 1% NEAA, 10% FCS, 0.5% Penicillin/streptomycin, 1% stable glutamine solution, 10 ng/mL EGF, 1 µg/mL Hydrocortisone)

Additional information

The general principle for cultivating spheroids is described in **001_Protocol_Cultivating_Spheroids_and_Organoids** with references to relevant protocols. The cells used for the procedure were 70-90 % confluent see protocol **002_Protocol_Sub-Cultivating_Cells_in_2D,** which describes sub-cultivation of cells, the protocol should be adjusted with the correct media composition described here. The day prior to the experiment initiate the equilibration of the ClinoReactor® see protocol **003_Protocol_Preparation_of_ClinoReactor**. Media change procedure is described in **004_Protocol_Cell_Culture_Media_Change_ClinoReactor**.

Protocol

<u>Preparation of initial 2D cell culture</u>

1. HMEC1 (ATCC, CRL-3243) was thawed in Complete MCDB131 media.
2. The cell suspension of HMEC1 cells was seeded into a T25 flask.
3. Complete MCDB131 media for flat culture was changed every two to three days.
4. When cells reached a confluence of 60-90 % the cells were sub-cultured
    a. Media was removed from the flask.
    b. The cell surface was washed two times with Hanks basal salt solution (HBSS) without calcium and magnesium.
    c. 9 mL of HBSS was added to one mL of trypsin/EDTA 10X to create a working solution.
    d. 3 mL of trypsin working solution was added and the flask was placed in the incubator for 3 minutes.

e. 1 mL of FCS was added to the flask to stop the trypsin activity.
f. The liquid was removed from the flask into sterile 12 mL centrifuge tube.
g. The flask was washed with 3 mL of Complete MCDB131 media, which was also transferred to the same centrifuge tube.
h. The cell suspension was centrifuged at 120 xG for 5 minutes. The supernatant was removed and 5 mL of Complete DMEM media was used to re-suspend the cells.
i. C3A cells can be diluted up to 1:6 and HMEC1 1:12, depended on the original confluence of the sub-cultured flask.

<u>Transfer of cells to low adherent plates.</u>

1. Spheroids were created by making cell composite aggregates of 20% HMEC1 cells where the remaining was HEPG2/C3A cells.
2. AggreWell™400 wells were washed with Complete DMEM media, and the liquid was removed.
3. 0.5 mL of rinsing was added to the wells.
4. The AggreWell™ plate was spun down at maximum RCF for 5 minutes.
5. The rinsing solution was removed, and the wells were washed with 1 mL Complete DMEM media.
6. 0.5 mL of Complete DMEM media was added to wells and the AggreWell™ plate was spun at maximum RFC for 9 minutes to remove bubbles.
7. The AggreWell™ plate was placed in the incubator until use.
8. Two at least 60 % confluent T75 C3A flasks and one at least 60 % confluent HMEC1 flask was sub-cultured as described in the section above.
9. The cells were diluted 1:20 in HBSS without calcium and magnesium and counted using Scepter cell counting (Millipore).
10. A thousand cells were seeded per microwell (each AggreWell™ contain 1200 microwells). The cells were seeded in 20 % HMEC1, 80 % C3A)
11. After appropriate incubation time (overnight) the aggregates were removed from AggreWells™ with a cut 1 mL tip and transferred to a petri dish with plenty of preheated media.
12. The aggregates were observed under inverted microscope and any clumps or non-homogeneous aggregates were discarded.

<u>Culturing spheroids in the ClinoReactors</u>

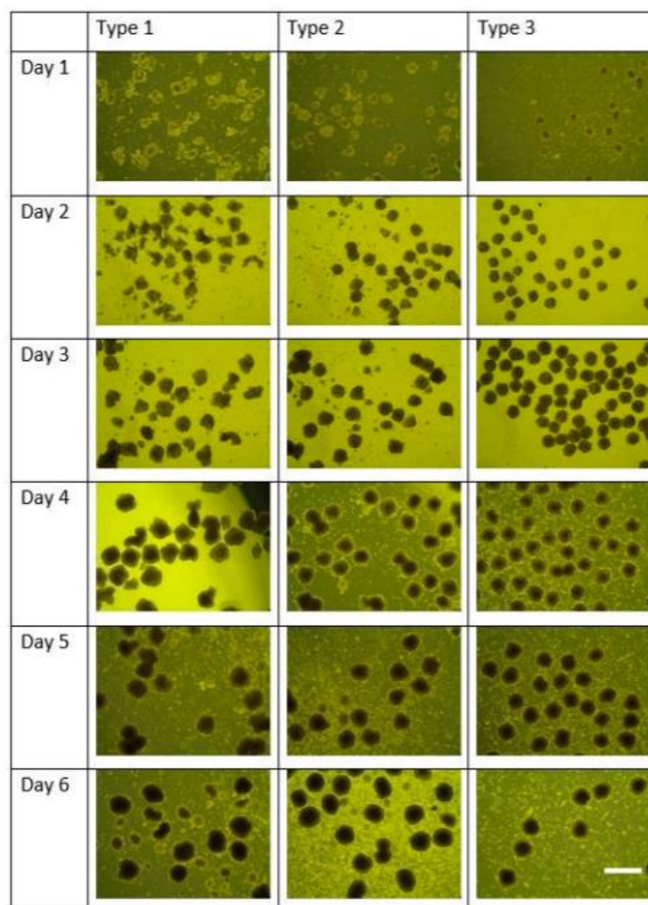
1. The cellular aggregates were transferred to the ClinoReactors®.
2. Fresh pre-heated Complete DMEM media was added carefully with a syringe (10 ml)) and needle 0.8 x 50 mm to maximum capacity to avoid bubbles in the cell chamber.
3. Any bubbles or clumps present were removed to avoid excessive oxidation and to maintain only single spheroid structure.
4. The spheroids were monitored, and their rotational speed adjusted to counteract their increasing size.
5. Media was changed following the 48-48-72 hours scheme.
6. To change the media, the bioreactor rotation was stopped so spheroids could fall to the bottom of the bioreactor.
7. Cell chambers were opened by the top plug and approximately 9 mL of Complete DMEM media was aspirated with syringe and needle.
8. New pre-heated media was carefully added to the bioreactor without disturbing the spheroids. Media was added to the very top to avoid bubbles in the cell chamber.
9. During the growth of spheroids or organoids it might be necessary to remove constructs with irregular shape or size to ensure optimal growth. It is recommended to do so during media exchange procedure but might be necessary to perform this removal ("gardening")

more frequently. Gardening is described in **006_Protocol_Gardening_Spheroids_or_Organoids.**

10. As the spheroids or organoids increase in size splitting the cultures into several vessels can be required to ensure optimal growth conditions. The procedure is described in **005_Protocol_Splitting_Spheroids_or_Organoids** and typically for immortal cells it should be done after approximately 7-10 days of growth.
11. Repeat step 9 and 10 as often as necessary.
12. The disposable ClinoReactor® vessel should be replaced every 10 days. The procedure is described in **007_Transferring_Spheroids_or_Organoids.**

Progress of spheroids

Below is shown how spheroids develop over time in the ClinoReactors. Type 1 is 0 % HMEC1 cells and type 2 is 20% HMEC1 and 80% HEPG2/C3A cells as described in this protocol. Finally type 3 is 40% HMEC1 cells and 60% HEPG2/C3A cells. For performing 40% HMEC1 cells this protocol can be followed with only adjusting the ratio between HEPG2 and HMEC1 cells.



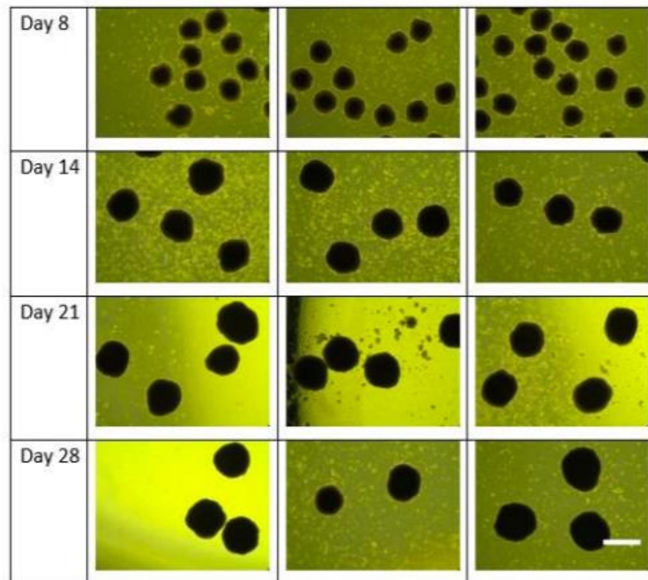


Figure 1 Long-term culture of type 1, 2 and 3 spheroids (day 8-28) Pictures show how type 1 (0 % HMEC1 cells), type 2 (20 % HMEC1 cells) and type 3 (40 % HMEC1 cells) spheroids look during 28 days of culture in bioreactors. Scalebar equals 1 mm.

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