Cell Culture Protocol

Using the Spheroid Kit with A549 or HepG2

This is a suggested procedure, please adjust according to your experimental needs. To maintain the sterility of the product, work under sterile conditions.

Protocol aim

The aim of this protocol is to provide instructions for culturing A549 cells in a Sphericalplate 5D® in order to form spheroids before bioprinting. This document covers the handling of A549 cells before and after seeding on the Sphericalplate 5D as well as the harvesting of the spheroids. Also covered is how to mix the spheroids with GelMA or Coll 1 for bioprinting or dispensing.

This protocol can be performed using human hepatocellular carcinoma cells HepG2 by substituting the medium MEM (Gibco, Ref#51200-046) with 10% FBS, 1% GlutaMAX (Gibco, Ref#35050-061), 1% NEAA (Gibco, Ref#11140-035), 1% sodium pyruvate (Gibco, Ref#11360-070), 0.5% Anti-Anti (Gibco, Ref#15240-062) and 0.5% PEST.

Materials needed

- Spheroid Kit (GelMA or Coll 1)*
- Carcinomic human lung epithelial cells A549 (in culture)
- RPMI 1640 medium (Gibco, Ref#22400-089)
- Fetal bovine serum (FBS) (Gibco, Ref#10270-106)
- Penicillin-streptomycin (PEST) (Gibco, Ref#15140-122)
- TrypLE Express (Gibco, Ref#12563-029)
- Phosphate-buffered saline (PBS) (Gibco, Ref#10010-015)
- Cell counter
- Microscope
- Lab consumables
- Bioprinting consumables*

*These products can be purchased from CELLINK at *www.cellink.com/store*.

Protocol

Before starting this protocol, make sure to have A549 cells in culture ready to be harvested.

Step	Title	Material	Description
1	Prepare medium	- RPMI 1640 medium - FBS - PEST	Prepare medium by mixing RPMI 1640 with 10% FBS and 1% PEST. This medium is now the complete culturing medium for A549.
2	Prepare cell suspension	- Confluent A549 in culture - PBS - TrypLE Express - Complete medium - Cell counter	Prepare a cell suspension by detaching the confluent A549 cells from the culture vessel. - Aspirate the medium in the flask and add PBS to cover the bottom of the flask. - Aspirate the PBS and add 0.04 mL TrypLE Express/cm^2 flask bottom area. Place in 37°C incubator for 5 minutes or until most of the cells have visibly detached. Gently tap the flask to detach all cells. - Add equal volume of complete medium to the flask and transfer the solution to a Falcon tube. Centrifuge at 112 g for 3 minutes to pellet the cells. Remove the supernatant and resuspend in 5 mL of new complete medium. - Count the cells in the suspension using a cell counter.
3	Seed on Sphericalplate 5D	- A549 in suspension - Complete medium - Sphericalplate 5D	- Pre-wet the Sphericalplate 5D wells by adding 0.5 mL medium to all wells used for this experiment and PBS to the rest of the wells. Centrifuge for 2 minutes at 1,000 rpm to remove air bubbles. - Account for 100 cells/microwell which results in 75,000 cells/well. Calculate how much cell suspension to add to each well. - Add the calculated amount of cell suspension to each well with medium and fill up to 1-2 mL of complete medium. Leave the unused wells filled with PBS. - Place the plate in the incubator in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity). - Be careful not to move or agitate the plate during culture. Note: Increase or decrease the initial cell seeding density according to experimental needs. When using the remaining wells, remove the PBS and continue adding the medium

			and cell suspension. No further centrifuging is needed.
4	Spheroid culture	- Microscope - Complete medium	- Monitor the spheroids by carefully transferring the plate to the microscope. - Monitor the size and shape of the spheroids each day until desired size and shape are visible, approximately 2 to 6 days. - Also monitor the color of the cell medium (if using the phenol red indicator). Preferably, avoid changing the medium if culturing for less than 3 days. Adjust the medium change according to culture needs. - If medium needs to be changed, carefully remove the upper half of the medium without tilting the plate or disturbing the spheroids. Gently add 1 mL of new complete medium by adding it slowly to the side of the well. The addition of 1 mL should take approximately 30 seconds. Note: If the plate is tilted during movement, the spheroids can be displaced and will no longer grow uniformly.

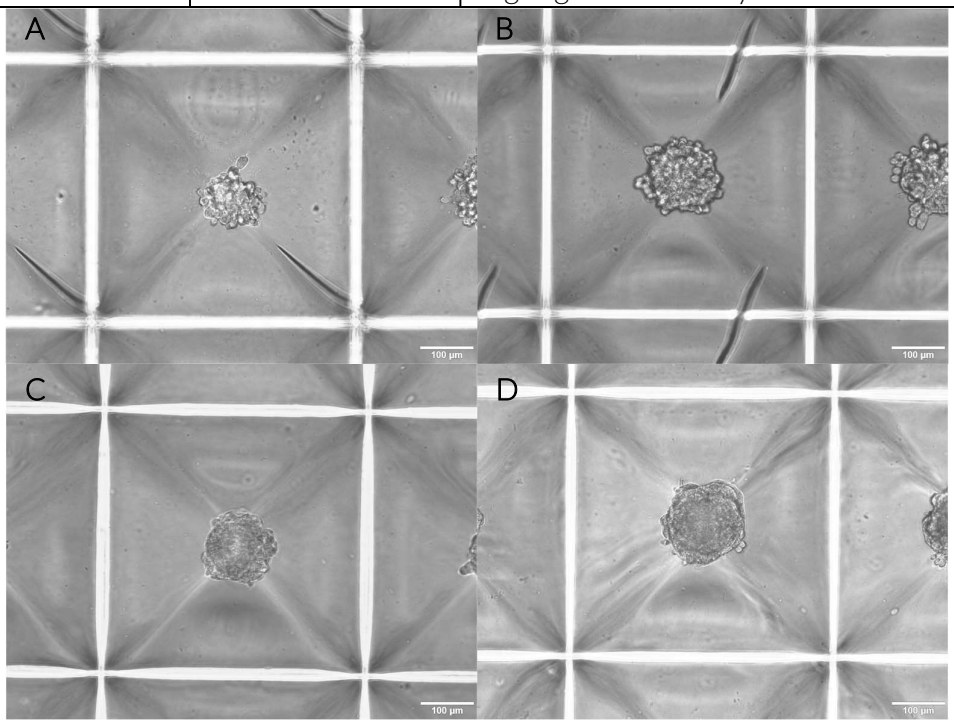

Figure 1. *A549 after seeding 100 cells/microwell A) Day 2, B) Day 3, C) Day 5 D) Day 7. Scale bar = 100 µm.*

5	Harvesting of spheroids	- Sphericalplate 5D with spheroids - Complete medium - 1 mL pipette tips	When spheroid size is as desired, harvest the spheroids. - Transfer half of the medium in a well to a tube. Tilt the plate and wash the microwells with the remaining medium a few times using a 1 mL pipette tip. Take medium from the bottom of the plate, still tilting, and transfer to the tube. Keep washing with new medium until all spheroids are harvested. - Let the spheroids sediment in the tube or centrifuge gently. Remove the supernatant and use the spheroids for the desired application. Note: When harvesting the spheroids, the plate needs to be tilted, a process that can displace all spheroids on the plate from their original microwells. Plan to harvest all wells on the same day for best results.
6	Bioprinting with spheroids	- Spheroids in suspension - CELLINK GelMA or Coll 1 - 3 mL syringes with Luer lock connections - Female/female Luer lock adapter - Cartridge, 3cc - Sterile conical bioprinting nozzles, 18-22G - BIO X 3D bioprinter - Temperature-controlled Printhead	- Heat GelMA to 37°C or prepare Coll 1 according to protocol. - Transfer 2.5 mL of bioink to a 3 mL syringe. Add 250 µL of the spheroid suspension with desired number of spheroids to the bioink syringe using a large pipette tip. - Connect the syringe with a second syringe using a Luer lock connector and gently mix back and forth a few times until the spheroids are homogeneously distributed in the liquid bioink. - Transfer the mixture to a 3cc cartridge for bioprinting and cap with a tip cap. - Gently flip the cartridge back and forth a few times until the bioink has set slightly to ensure homogeneous distribution of the spheroids. The spheroids can sediment if they are placed upright in the printhead in liquid bioink and left idle for a few minutes. - See Bioprinting Protocol for GelMA or Casting Protocol for Coll 1 for more details on the printing/dispensing procedure. Print using a large nozzle to avoid disrupting the spheroids in the printing process. Note: If the bioink is completely liquid after the addition of spheroid suspension, tilting the syringe back and forth can be enough to distribute the spheroids evenly within the bioink.

Troubleshooting guide

The spheroids are of different size.

- If the plate is moved or agitated during the first couple of hours after seeding, the cells can sediment unevenly or move to neighboring microwells. Avoid moving the plate within the first 12 hours after seeding.

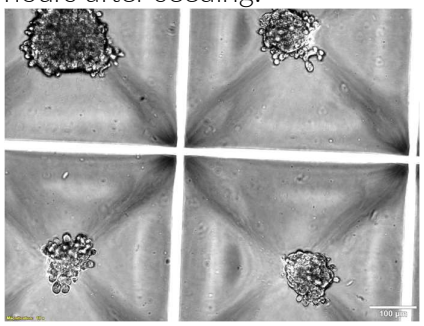

Figure 2. Deviation when cells move into other microwells can lead to the growth of non-uniform spheroids. Scale bar = 100 µm.

The spheroids moved into the same microwell.

- If the plate is tilted or moved harshly, the spheroids can move into neighboring microwells. Keep the plate in an upright position and move with a steady hand.
- Changing medium can stir up the spheroids and have them settle down in another microwell. Change medium only if needed and by gently removing and adding new medium.
- Having 2 mL medium in each well can help prevent the spheroids from moving.

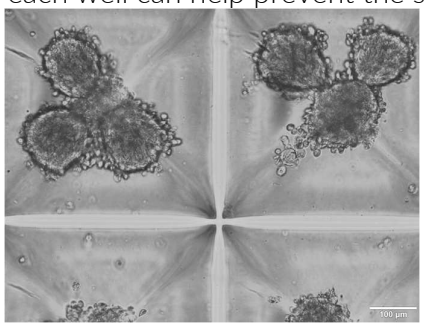

Figure 3. Deviation when spheroids move into other microwells and form clusters. Scale bar = 100 µm.

The spheroids are larger on one side of the well and smaller on the opposite side.

- Cells can sediment more toward one side if the plate has tilted during the first hours after seeding. Make sure to place the plate on a leveled shelf in the incubator.

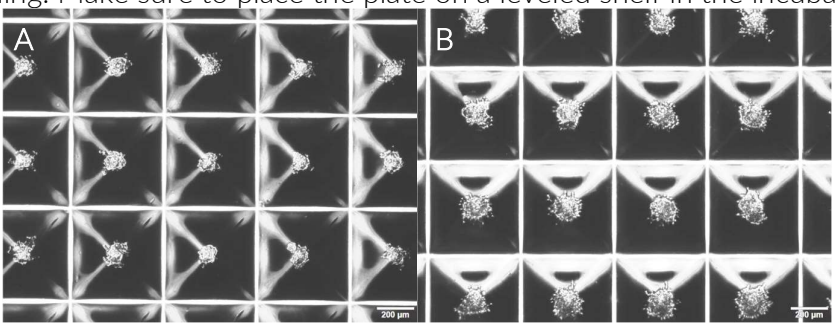

Figure 4. Deviation when spheroids are A) smaller on one side of the plate and B) larger on the opposite side. Scale bar = 200 µm.