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TECHNICAL PROTOCOL

3D Aggregation of Tumor Spheroids in the Akura™ 384 Spheroid Microplate

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InSphero Akura™ 384 Spheroid Microplates represent a simple, flexible, and automation-compatible platform for the generation, long-term cultivation, observation, and testing of scaffold-free 3D cell models in a 384-well format. Each plate features a special ultra-low attachment (ULA) surface, is sterile-packaged, and provided with a lid. The unique well geometry of the Akura™ 384, comprised of a SureXchange™ ledge and dedicated spheroid compartment, ensures precise and accurate media exchange without spheroid loss, and low residual volumes. The Akura™ 384 Spheroid Microplate is also compatible with a broad spectrum of assay endpoints and uniquely designed to support high resolution fluorescent imaging of spheroids.

The following technical protocol describes a method for generating 3D tumor spheroids using the Akura™ 384 Spheroid Microplate. The goal of this technical protocol is to provide step-by-step instructions for the successful aggregation of 3D spheroids and to enable a quick and productive first experience with the Akura™ Plate technology.

The technical protocol covers plate preparation, generation of the cell suspension for seeding, optimization of seeding density, tips and tricks to promote rapid, uniform cell aggregation, and suggested quality control steps.

AGGREGATION | Imaging | Handling

NCI-N87-GFP / NIH-3T3-RFP Spheroid Co-culture Formation – A Step-by-step Protocol

Materials

- Cryopreserved NCI-N87-GFP and NIH-3T3-RFP cells, ideally 1×10^6 cells per vial
- Cell-culture flasks T75 (Greiner, cat no. 658175)
- Akura™ 384 Plates (InSphero, cat no. CS-09-003-02)
- Sterile phosphate buffered saline without Ca++ and Mg++ (PBS w/o Ca++Mg++) (Sigma-Aldrich, cat no. D1408)
- Neubauer chamber
- Water bath (37 °C)
- Serological pipettes, 5 and 10 ml
- Centrifuge with microplate carriers
- Level 1 biosafety cabinet
- Humidified 5% CO_2 incubator 37 °C
- Inverted phase-contrast microscope
- 15 ml sterile Falcon tube
- Sterile multichannel medium reservoir
- Multichannel pipette (e.g. Integra Viaflo 8-channel pipette) and sterile tips
- **Optional:** Automated bright-field spheroid imaging system (e.g. SCREEN Cell^{3}iMager™)
- Fluorescence microscope or High Content Imaging instrument (e.g. Leica DMi8 or, Yokogawa CQ1)

NCI-N87-GFP and NIH/3T3-RFP Expansion

IMPORTANT:

- **Perform all following steps in a biosafety cabinet under aseptic conditions. Procedures are the same for both NCI-N87-GFP and NIH-3T3-RFP cells except where otherwise indicated.**

1. Ensure that all cell-culture material is in place and labelled.
2. Pre-warm cell culture media to 37 °C.
3. Prepare T75 flask by adding 5 ml of pre-warmed medium.
4. Fast thaw one vial of each cell line at 37 °C in the water bath.
5. Using the 5 ml pipette, aspirate 5 ml pre-warmed medium from the media bottle.
6. Use the filled 5 ml pipette to aspirate the thawed cell suspension from the cryo vial and transfer 5 ml of the resulting mixture into a 15 ml tube. Rinse the cryo vial with the remaining 1 ml of medium from the pipette and add it to the 15 ml tube.
7. Centrifuge the cells at 200 RCF for 2 min, remove the supernatant and resuspend the cell pellet with 5 ml pre-warmed medium.
8. Transfer resuspended cells into the pre-filled T75 flask.
9. Place the cell-culture flask into the incubator.
10. After 24 hours of incubation replace the medium and check under the microscope if cells have adhered on the plastic surface.
11. After reaching 70-80% confluence (approx. 48 hours) cells are ready for spheroid production.

NCI-N87-GFP/NIH-3T3-RFP aggregation in the Akura™ 384 Plate

IMPORTANT:

- **Pre-wetting the wells of the Akura™ 384 Plate according to the procedure below is required prior to seeding spheroids to prevent formation of air bubbles during aggregation.**
- **The Akura™ 384 Plate has an ultra-thin 25 µm foil bottom, avoid contacting the bottom with pipette tips as the bottom can be easily punctured or deformed.**
- **Perform the following steps under sterile conditions.**

Pre-wetting

1. Add 50 µl of PBS to each well by placing the tip near to, but not touching the bottom of the well. It is recommended to use a multichannel pipette (8- or 12-channel).
2. Centrifuge the Akura™ Plate for 2 minutes at 250 RCF and incubate it in a humidified CO_2 incubator for at least 1 day.
3. Before cell seeding take the Akura™ Plate from the incubator, centrifuge the Akura™ Plate for 2 minutes at 250 RCF. Aspirate the pre-wetting solution by placing the tip at the ledge of the upper cavity of the well. Aspirate until the PBS is removed from each well. A small amount of PBS (< 2-3 µl) remains in the bottom of the chamber.

Preparing the Cell Suspension

1. Prior to seeding, pre-warm the cell maintenance medium to 37°C.
2. Take the T75 flasks with the NCI-N87-GFP or NIH-3T3-RFP cells out of the incubator.
3. Remove medium with the aspiration pipette.
4. Add 10 ml PBS w/o Ca++ Mg++ and gently tilt flask back and forth.
5. Remove PBS.
6. Add 1 ml Trypsin EDTA (1×).
7. Incubate at 37 °C for 5 minutes.
8. Ensure that the cells are completely detached.
9. Stop trypsinization by adding 9 ml of medium containing FBS.
10. Transfer the cell suspension into a 15 ml Falcon tube.
11. Centrifuge for 2 minutes at 200 RCF.
12. Aspirate supernatant.
13. Re-suspend cells in medium depending on cell pellet size.
14. Determine cell number with the Neubauer chamber (or alternative method).
15. Dilute the NIH-N87-GFP cells to a density of 500 cells/50 µl and NCI-3T3-RFP cells to a density of 3000 cells/50 µl. Seed them together with a cell suspension volume sufficient for the number of desired replicates.
16. Prepare about 30 ml of cell suspension to seed the whole plate with a multichannel pipet or a 384w pipet-head on a automated liquid handling system.

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Cell Seeding

IMPORTANT:

- To enable direct comparisons between spheroid treatment groups the spheroids should be uniform in size and cellular composition at the onset of the study.
- To ensure uniformity, begin with a well-dispersed cell suspension.

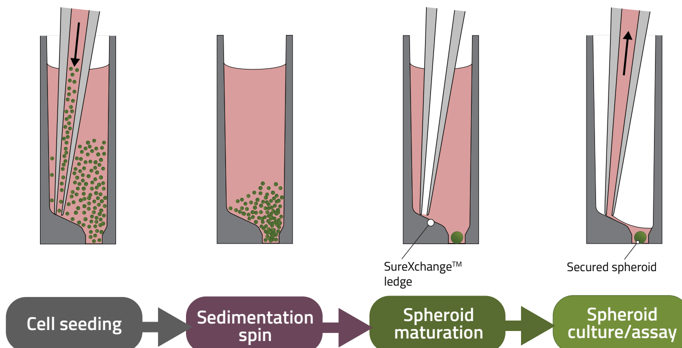

Figure 1: Cartoon representation of pipetting operations performed in the Akura™ 384 Plate, including cell seeding, spheroid aggregation, and media withdrawal.

1. Transfer cell suspension to a medium reservoir. Obtain a homogeneous cell distribution by gently pipetting up and down prior to seeding into the Akura™ 384 Plate. Seed 50 µl of cell suspension/well using a multi-channel pipette.
2. Place lid on bottom plate and centrifuge the Akura™ Plate for 2 minutes at 250 RCF.
3. Tilt plates to approximately 30° by leaning the plates against another plate or by placing the plate on the specially designed Akura™ Tilting Stand (InSphero, CS-10-002-00). Incubate them in a humidified CO_2 incubator for 3 days and inspect the wells for spheroid formation using an inverted microscope equipped with a 5-10X objective.
4. As an additional quality control step, spheroid morphology and viability can be verified by brightfield imaging (e.g SCREEN Cell3iMager), fluorescence imaging (e.g. Leica DMi8, Yokogawa CQ1) and/or a cell viability assay (e.g. Promega CellTiter-Glo Assay Kit).

Results

The scaffold-free gravity-assisted aggregation of tumor and fibroblast cells in the Akura™ 384 Plates via the method provided above generates compact fully formed spheroids of uniform size and cellular composition (*Figure 2*), a prerequisite for high throughput screening. The 384-well plate technology is compatible with both liquid handling devices (*Figure 1*) and automated high content imaging on the Yokogawa CV8000, and therefore meets the minimum criteria for an imaging-based screening campaign.

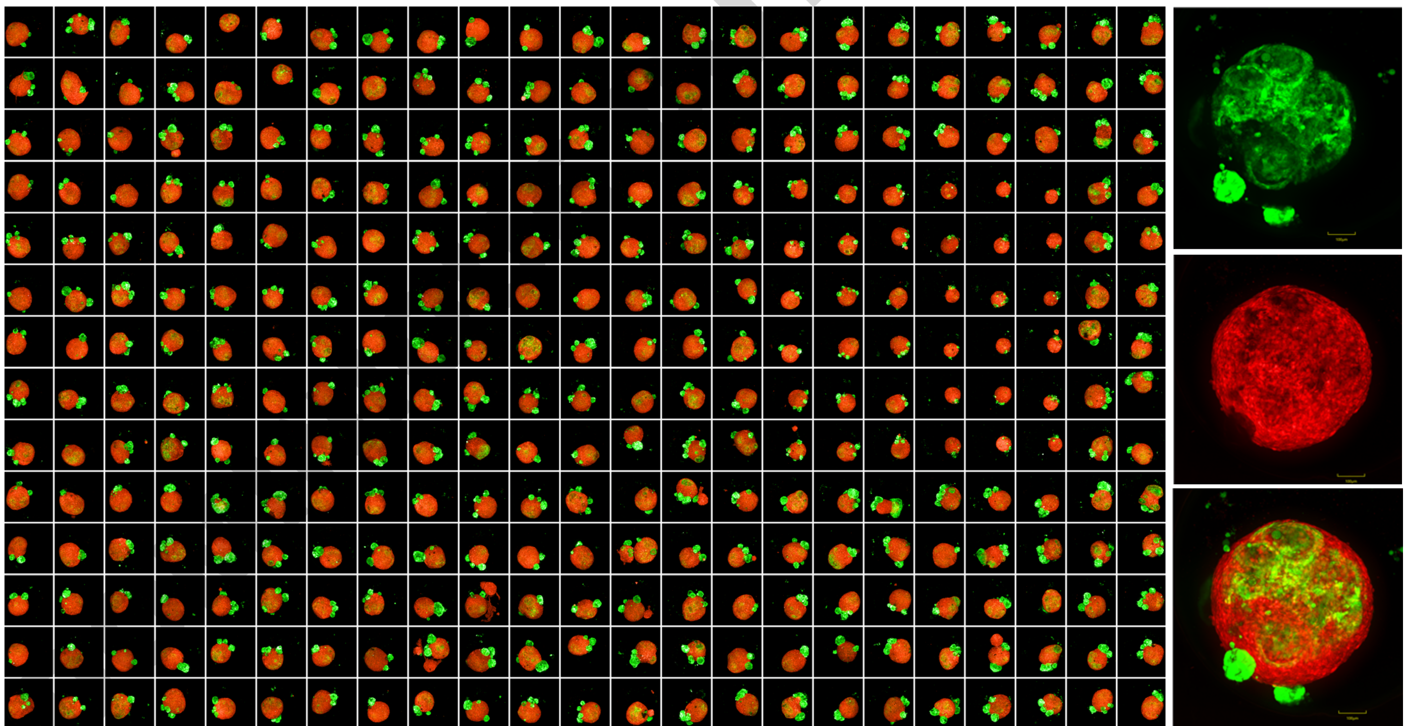

***Figure 2:** Left panel- Maximum intensity projection images (MIPs) from 322 wells of an Akura™ 384 Plate. Each well contains a single tumor spheroid comprised of a NIH-N87-GFP (tumor cells) and NIH3T3-RFP (fibroblasts) co-culture. Following aggregation, the tumor spheroids were maintained in culture media either with or without compound for an additional 7 days and then fixed and imaged on a Yokogawa CV8000 confocal high content analysis instrument. Wells treated with increasing concentrations of Lapatinib, a targeted tumor therapy, are indicated by the yellow bounding box, all other wells are untreated. Right panel- A set of red, green and composite images acquired from a single untreated well, enlarged to illustrate image resolution.*

CONCLUSION

The Akura™ 384 Spheroid Microplate is designed for the routine generation and long-term maintenance of spheroids and for automated high throughput applications. The inert, ultra-low attachment surface ensures that spheroids do not adhere to plastic during production or culture and the unique SureXchange™ ledge enables > 90% media exchange while protecting the spheroid from accidental aspiration or damage.

The plate material is compatible with most solvents and therefore a variety of endpoint assays can be performed directly in the Akura™ 384 Spheroid Microplate. Compliance with ANSI/SLAS microplate standards also ensures compatibility with automation and straightforward integration of the Akura™ 384 Spheroid Microplate into standard HTS workflows. The flat, transparent bottom also enables both bright field and epi-fluorescent imaging on a variety variety of microscope and high content imaging platforms.

Discover more and speed up your workflow:

Semi-automated medium exchange in the Akura™ 384 Spheroid Microplate with the INTEGRA VIAFLO 384

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