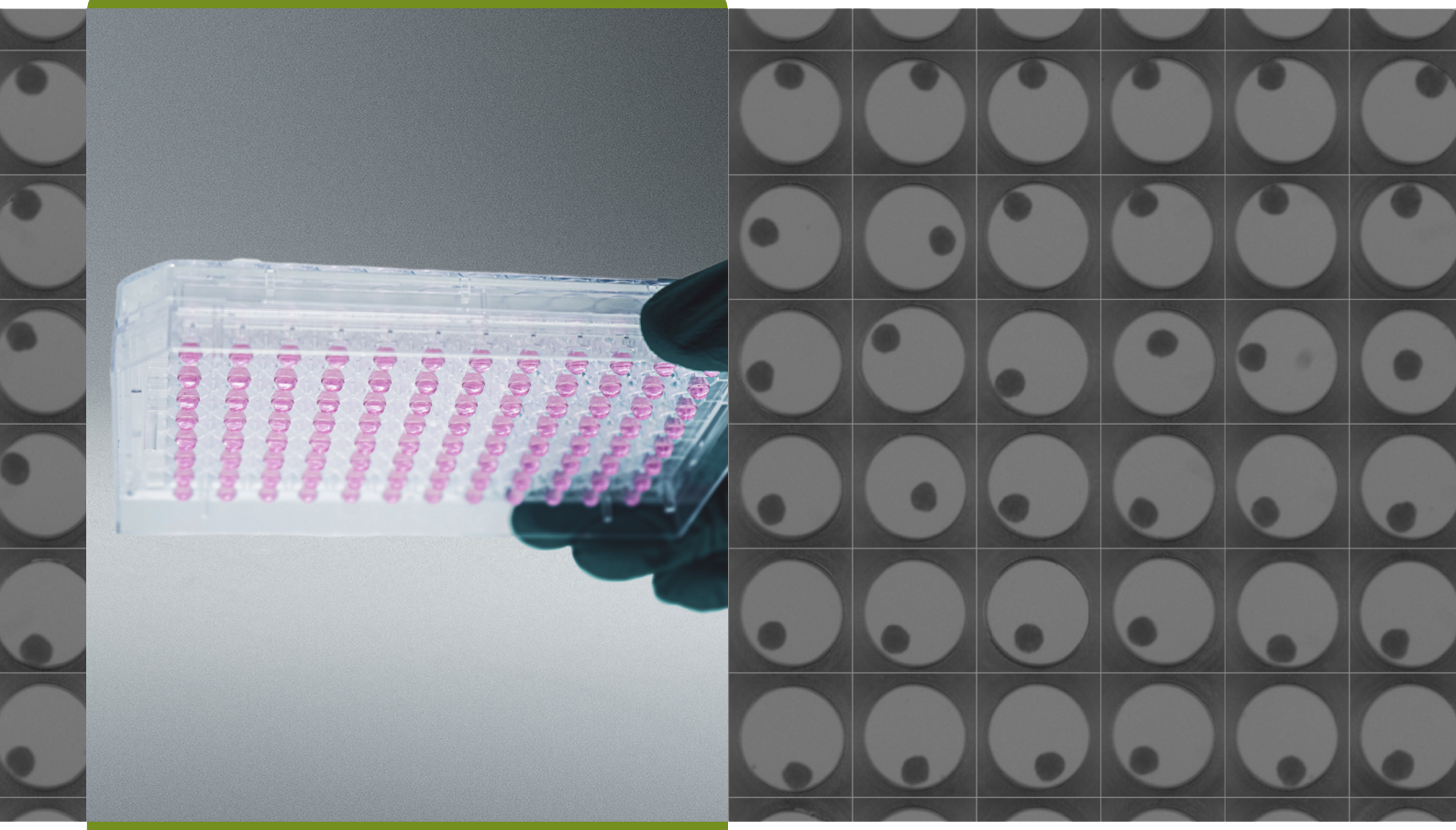

TECHNICAL PROTOCOL

3D Aggregation of NIH3T3 Fibroblasts using the Akura™ PLUS Hanging Drop System

The Akura™ PLUS Hanging Drop System is a thoughtfully engineered system that can be used for the reproducible, reliable production and long-term maintenance of 3D spheroids. The spheroids produced in the Akura™ PLUS Hanging Drop System are uniform in size and cellular composition, and hourglass geometry promotes stability of the droplet. The unique well geometry of the Akura™ 96 receiver plate, comprised of a SureXchange™ ledge and dedicated spheroid compartment, ensures precise and accurate media exchange, as well as low residual volumes.

The following technical protocol describes a method for generating 3D tumor spheroids using the Akura™ PLUS Hanging Drop System. The goal of this technical protocol is to provide step-by-step instructions for successful spheroid generation and to enable a quick and productive first experience with the Akura™ PLUS Hanging Drop System.

This technical protocol covers preparation of the cell suspension, proper plate preparation, hanging drop formation, and transfer of spheroids to the Akura™ 96 Plate for long-term maintenance.

AGGREGATION

Imaging

Handling

Spheroid formation

The following protocol describes the production of NIH/3T3 (mouse fibroblast cell line) spheroids with the Akura™ PLUS Hanging Drop System.

Materials

- Cryopreserved NIH/3T3 mouse fibroblasts, ideally 1×10^6 cells per vial (ATCC CRL-1658™)
- Expansion medium: DMEM (PAA, cat. no. E15-883) supplemented with 10% FCS (PAA, cat. No. A15-151)
- Trypsin EDTA (10×) 0.5% Trypsin /0.2% EDTA in DPBS (PAA, cat. no. L11-003)
- Maintenance medium of choice
- Cell-culture flasks T75 (Greiner, cat no. 658175)
- Akura™ PLUS Hanging Drop System, CS-06-004-01
- Sterile phosphate buffered saline without Ca++ and Mg++ (PBS) (Sigma-Aldrich, cat no. D1408)
- Neubauer chamber
- Water bath (37°C)
- Serological pipettes, 5 and 10 ml
- Level 1 biosafety cabinet
- Humidified 5% CO_2 incubator 37°C
- Inverted phase-contrast microscope
- 15 ml Falcon tube
- Sterile multichannel medium reservoir
- Multichannel pipette (e.g. Integra Viaflo 8-channel pipette)

NIH/3T3 thawing and expansion

IMPORTANT:

- **Perform the following steps under aseptic conditions in a biosafety cabinet.**
- **Before beginning, ensure that all cell-culture supplies are in place and properly labelled.**

1. Pre-warm the DMEM + 10% FCS to 37°C.
2. Prepare T75 flask by adding 5 ml of DMEM + 10% FCS.
3. Quick thaw one vial NIH/3T3 at 37°C in the water bath.
4. Use the 5 ml pipette and aspirate 5 ml pre-warmed medium from the media bottle.
5. Use the filled 5 ml pipette and aspirate the thawed cell suspension from the cryo vial and transfer 5 ml into a 15 ml tube. Rinse the cryo vial with the remaining 1 ml medium in the pipette and add to the 15 ml tube.
6. Centrifuge the cells at 200 RCF for 2 min, remove the supernatant and resuspend the cell pellet with 5 ml of pre-warmed medium.
7. Transfer resuspended cells into the pre-filled T75 flask.
8. Place the cell-culture flask into the incubator.
9. After 24 hours of incubation replace the medium and check under the microscope if cells have adhered on the plastic surface.
10. After reaching 70-80% confluence (approx. 48 hours) cells are ready for microtissue production.

Akura™ PLUS Plate preparation

IMPORTANT:

- **Perform all following steps under aseptic conditions under a laminar flow bench.**

1. Wipe the Akura™ PLUS Plate bag with 70% EtOH before opening.
2. Carefully open the bag under sterile working conditions and take out the Akura™ PLUS Plate assembly.
3. Prepare a reservoir (e.g. a 15 cm diameter petri dish) with 20 ml 0.5x PBS.
4. Open the bag containing humidifier pads. Using the tweezer, remove one humidifier pad and place it in the dedicated reservoir containing the 0.5x PBS.
5. Wait until the humidifier pad is completely soaked with PBS (approx. 5 min).
6. While pad is soaking, open the Akura™ PLUS Plate package and remove the frame.
7. Place the soaked humidifier pad in the bottom plate of the Akura™ PLUS Plate.

NIH/3T3 spheroid formation

1. Take the T75 flask with the NIH/3T3 cells out of the incubator.
2. Remove medium with the aspiration pipette.
3. Add 10 ml PBS (without Ca++ and Mg++).
4. Remove PBS.
5. Add 2.5 ml Trypsin EDTA (1×).
6. Incubate at 37°C for 5 minutes.
7. Ensure that the cells are completely detached.
8. Stop trypsinization by adding 7.5 ml of maintenance medium containing FCS.
9. Transfer the cell suspension into a 15 ml Falcon tube.
10. Centrifuge for 2 min at 200 RCF.
11. Aspirate supernatant.
12. Re-suspend cells in medium depending on cell pellet size.
13. Determine cell number with the Neubauer chamber (or alternative method).
14. Adjust cell number with medium to a density of 1.25×105 cells/ml corresponding to 5000 cells/drop (40 µl); prepare a sufficient amount of cell suspension (5 ml per Akura™ PLUS Plate).
15. Transfer cell suspension to a medium reservoir. Obtain a homogeneous cell distribution by gently pipetting up and down prior to seeding into the Akura™ PLUS Plate.

Hanging-drop formation

IMPORTANT:

- **To generate spheroids with uniform size and cell composition, it is essential to assure a homogeneous distribution of the cells by gently pipetting up and down prior to the seeding into the Akura™ PLUS Plate.**

1. Gently deliver 40 µl of cell suspension into each well of the Akura™ PLUS Plate. Ensure a tight contact between the pipette tip and the well inlet by applying a slight pressure to form the SureDrop™ seal (*Figure 1*).

2. Place the lid on the Akura™ PLUS Plate.
3. Place the Akura™ PLUS Plate assembly in a humidified 5% CO_2 incubator at 37 °C.

IMPORTANT: Please handle the PLUS Plate carefully to avoid any loss of drops.

4. Assess spheroid formation regularly. After 4 days in culture most cell types re-aggregate and form a compact spheroid.

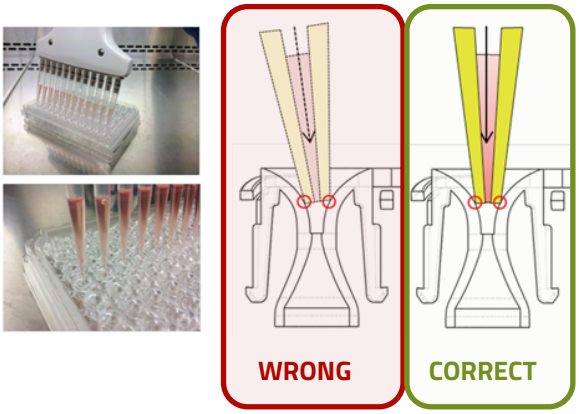

Figure 1: *Filling Akura™ PLUS wells. The pipette (8- or 12-channel) is positioned into the inlet of the well in an upright or slightly tilted orientation. It is important that the pipette tips make sufficient contact with the well surface to assure complete liquid transfer and uniform drop formation. The weight of the pipette alone is usually sufficient to provide adequate contact pressure.*

Long Term Cultivation and Assays

For long-term cultivation and assays, transfer of 3D cell culture models from the Akura™ PLUS Plate to the Akura™ 96 Plate is required.

Spheroid transfer to the Akura™ 96 Plate

1. Place frame with stripes of the Akura™ PLUS Plate onto the Akura™ 96 Plate (*Figure 3*) by positioning the three pins into corresponding holes on the top surface of the Akura™ 96 Plate (*Figure 2*). The drops under the Akura™ PLUS Plate will then be perfectly aligned with the wells of the Akura™ 96 Plate underneath.

Figure 2: *Akura™ 96 Plate – red circles indicating positioning pins for precise transfer of spheroids from the Akura™ PLUS Plate.*

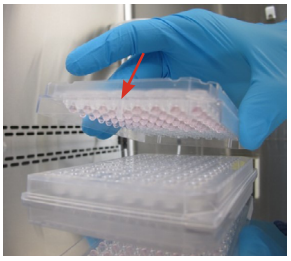

Figure 3: *Positioning three pins into corresponding holes on the top surface of the Akura™ 96 Plate.*

2. Slowly (i.e. ≤10 µl/sec when using Integra Viaflo pipettes) add 70 µl of medium through the inlet of the Akura™ PLUS Plate wells. The pipette tips should be in direct contact with the well inlets by simultaneously applying a subtle pressure with the pipette. The drops will fall into the Akura™ 96 Plate (*Figure 4*).

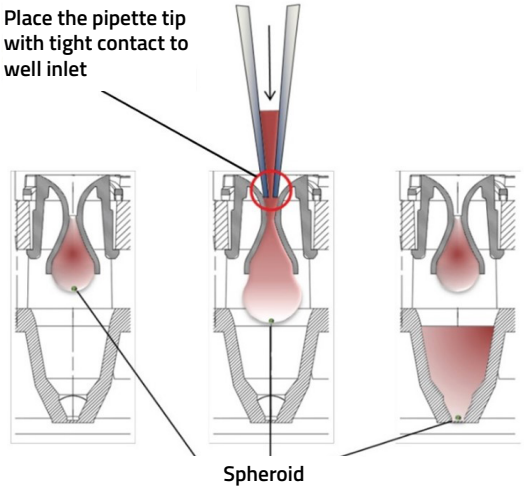

Figure 4: *3D cell culture model transfer from the Akura™ PLUS Plate into the Akura™ 96 Plate.*

3. Verify the transfer by a microscopic inspection of the wells (use an inverted microscope). After transfer of the spheroids/ organoids, place the Akura™ 96 Plate in a microplate centrifuge and spin for 2 min at 250 RCF. The centrifugation step will force the tissues to the bottom of the well and remove trapped air bubbles.
4. To assure defined medium volumes in the wells, the solution in the wells may be replaced by aspiration and addition of 70 µl of fresh medium (for more detailed information on spheroid maintenance including how to conduct medium exchanges without spheroid loss, please refer to the Akura™ PLUS Product Manual).

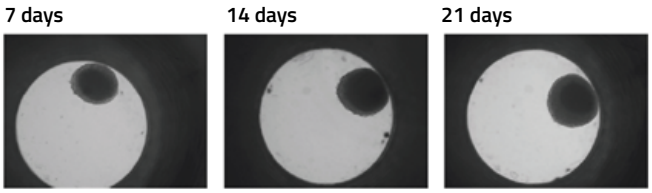

Figure 5: *NIH/3T3 spheroid stability over 21 days in the Akura™ 96 Plate.*

CONCLUSION

The Akura™ PLUS Hanging Drop System is an easy-to-use platform for the routine generation of spheroids with uniform size and cellular composition. The SureDrop™ seal and hourglass design of the Akura™ PLUS Plate ensures the generation of uniformly-sized and exceptionally stable hanging drops which can be incubated for several days enabling self-assembly and maturation of 3D spheroids. The Akura™ PLUS Hanging Drop Plate also mates seamlessly with the Akura™ 96 Plate for easy transfer of the spheroid-containing hanging drops to the ANSI/SLAS compliant ULA plate for long term culture.

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