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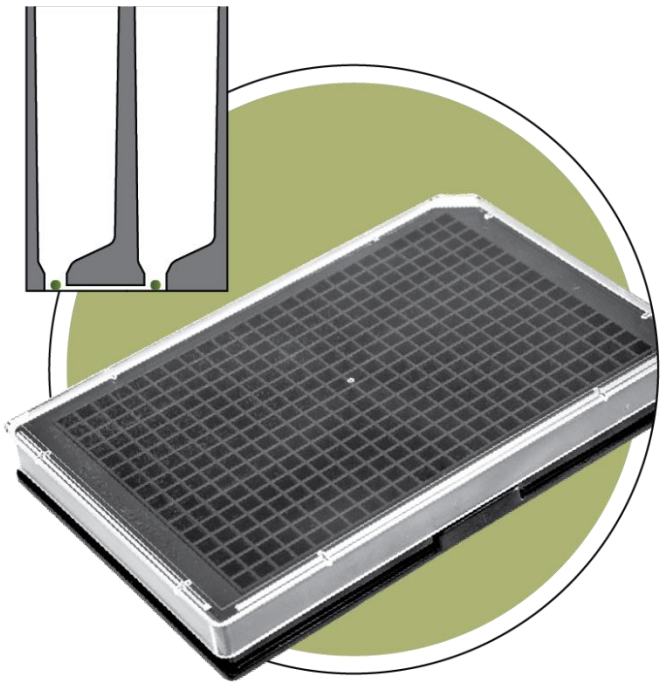
NEW

Akura™ Twin Microplate

Product Manual



PM0011, June 2024

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Introduction

InSphero's Akura™ 96 and 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 cultures – one spheroid/organoid per well.

The Akura™ Twin Microplate builds on the architecture of the Akura™ 384 Spheroid Microplate including all proven features. In addition, two wells are always interconnected by a microfluidic channel enabling cross-communication between two spheroids cultured side-by-side with or without immune cells.

Each plate consists of a sterile-packaged Akura™ Twin Microplate and lid. All wells and channels feature a special ultra-low attachment (ULA) coating. The Akura™ Twin Microplate is compatible with state-of-the-art imaging and automated liquid handling systems for HTS applications. Biochemical assays can also be performed on spheroids directly in the Akura™ Twin Microplate.

The Akura™ Twin Microplate is designed to be used with several 3D cell culture model types, such as organoids, spheroids and microtissues. For the remainder of the document, we will refer to these models as '**spheroids**'.

The Akura™ Twin Microplate is an assay plate for pre-formed spheroids. InSphero recommends Akura™ 96 and 384 Microplates for the generation of spheroids and parallel transfer into the Akura™ Twin Microplate after quality control of spheroids.

Design of the Akura™ Twin Microplate

The Akura™ Twin Microplate complies with the ANSI/SLAS 384-well plate standard format. It uses the same black plate body as the Akura™ 384 Spheroid Microplate. The tapered wells feature a SureXchange™ ledge to prevent inadvertent cell or spheroid aspiration and disruption during medium exchange and compound dosing. Spheroids are positioned in a 1.0 mm observation compartment at the bottom of each well, which enables automated imaging processes.

Two adjacent wells are always connected by a 90 µm high, 1.6 mm wide microchannel resulting in 192 twin units per plate. The orientation of the channels is horizontal for rows A and P and vertical for rows B to O. The low height of the microchannels retains spheroids larger than 100 µm in their wells, while soluble factors and suspended immune cells can move between the wells.

All well and microchannel surfaces feature cell-repellent properties for long-term, non-adherent culturing of spheroids and suspension cells.

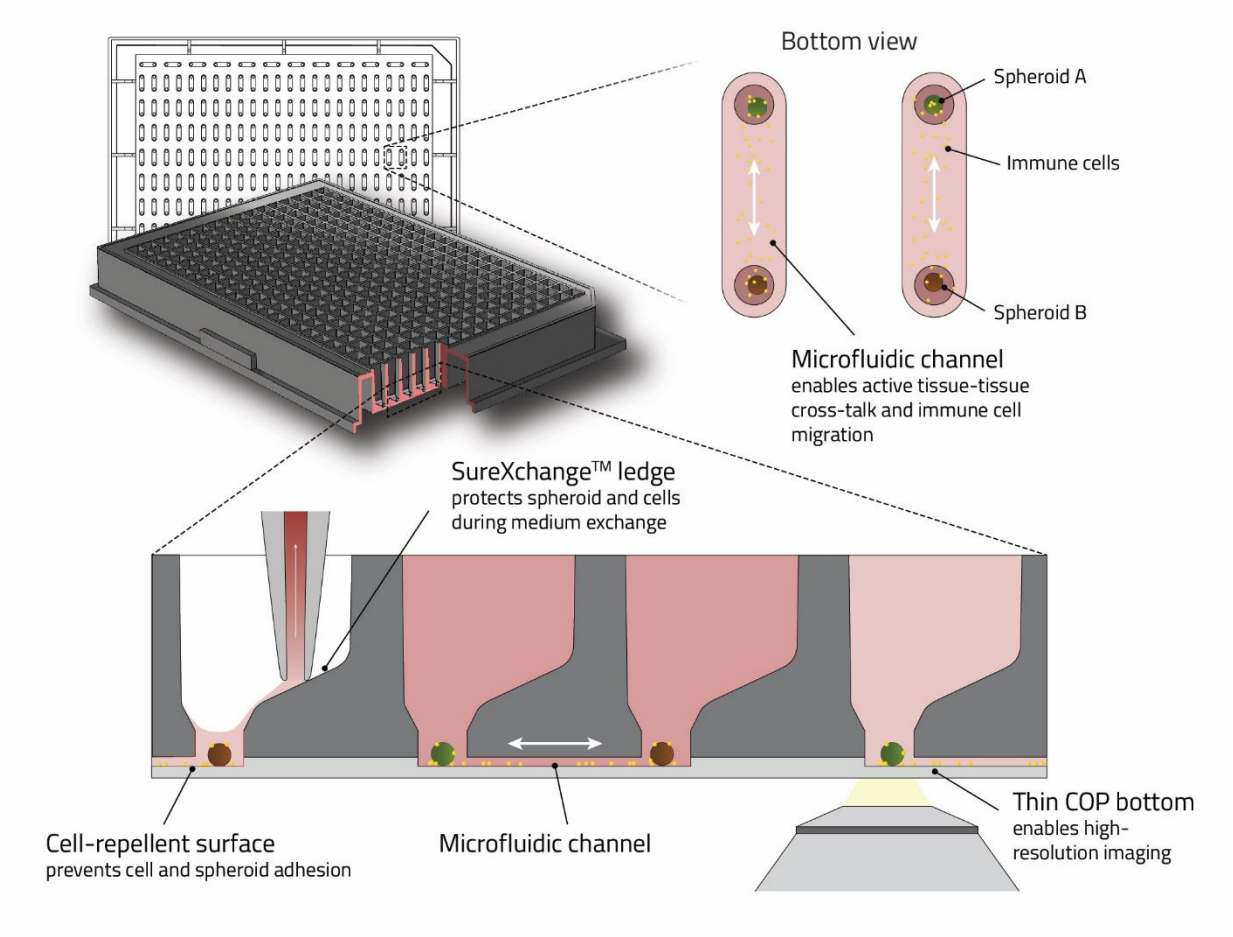


Figure 1: Illustration and enlarged cross-sectional view of the Akura™ Twin Microplate, highlighting the main features of the plate. (Please refer to Technical Specifications for more details.)

The 188 µm thick continuous COP (cyclo-olefin polymer) bottom of the plate enables high-resolution brightfield and fluorescence imaging of spheroids and cells in the wells and microchannels, respectively (Figure 1).

Unique features of the Akura™ Twin Microplate

- 1-to-1 interconnected wells for studying organ-organ crosstalk without losing single spheroid resolution during analysis.
- Up to 192 twin-organ conditions in a single plate to compare many replicates in parallel and reduce cost and effort per condition through scale up.
- SureXchange™ tapered ledge and culture compartment facilitates easy medium exchange and prevents spheroid and cell loss during long-term experiments and analysis.
- 1-mm diameter flat bottom observation compartment enables simple spheroid localization, observation, and ROI identification.

- Continuous, 188 µm COP bottom results in high imaging quality and the black-walled body eliminates fluorescent crosstalk between wells.
- Akura™ Twin Microplate format is compatible with state-of-the-art imaging and automated liquid handling systems enabling HTS applications.

Overall workflow of co-culturing experiments

Co-culturing experiments require a well-orchestrated experimental workflow to produce reproducible and robust results (Figure 2). This includes control over all relevant elements, including (a) viability and function of the 3D tissue models and cells, (b) availability of media, supplements, and compounds, and (c) preparation of plates, operational systems, and analytical instruments.

The Akura™ Twin Microplate is engineered for spheroid organ models, which must be produced in another plate prior to transferring them for co-culturing into the Akura™ Twin Microplate. We recommend the use of Akura™ 96 or 384 Microplates and protocols provided by InSphero AG. Alternatively, pre-formed, Assay-Ready Microtissues can be purchased at InSphero AG. In parallel, immune cells or other suspension cells of interest need to be prepared.

Individual production and preparation of tissue and cell models allow you to adhere to tissue-specific production protocols including special medium formulations, maturation timelines and pre-treatments. The different organ models are then transferred into the Akura™ Twin Microplate to start the co-culturing experiment. A preceding individual quality control step ensures robust system performance and reduces variability between replicates.

Akura™ Twin Microplates are incubated on an All-in-One Programmable Tilter rocking the plates back and forth with a defined sequence. The tilting induces gravity-based flow between the connected wells, resulting in continuous crosstalk. Tilting parameters can be programmed depending on the experimental objectives. Plates can be removed anytime for media exchange, media sampling and compound dosing. Furthermore, monitoring of cells and spheroids can be done through microscopy.

At the end of the experiment, spheroid models and cells can be removed for downstream analysis. Alternatively, cells and spheroids can be stained and cleared inside the plate for in-situ high-resolution microscopy.

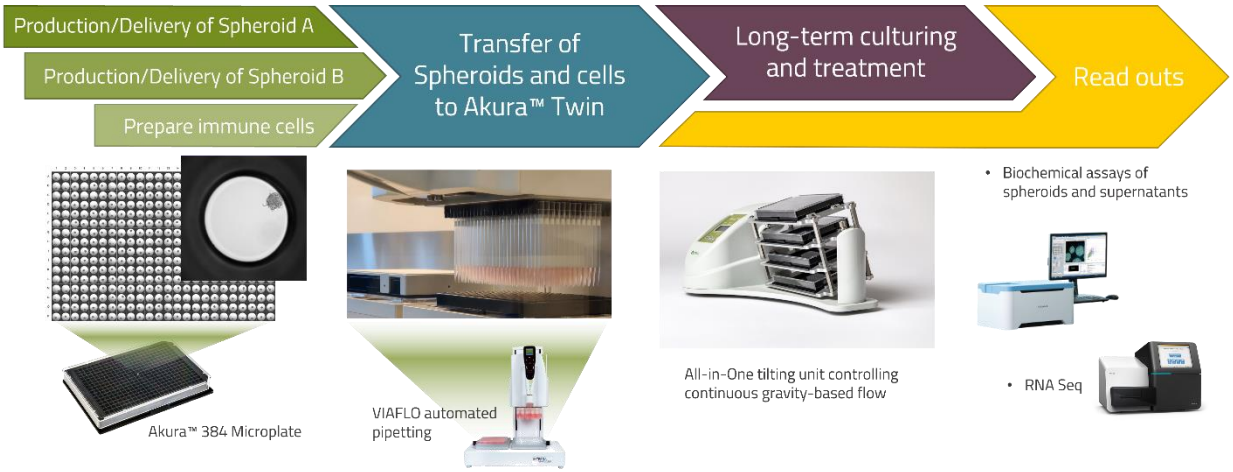


Figure 2: General workflow of co-culturing experiments in the Akura™ Twin platform.

Checklist for successful co-culturing experiments

- ✓ Organ models can be produced as scaffold-free spheroids in Akura™ 96 or 384 Microplates.
- ✓ Organ models show a compact morphology for pipette transfer with a diameter of more than 100 µm and less than 800 µm over the duration of the experiment (considering proliferation and growth).
- ✓ Quality control criteria for organ models have been established to assess functionality prior to co-culturing experiments.
- ✓ Common medium formulation for co-culturing has been established and preserves the relevant features of the individual organ models.
- ✓ Protocols for immune cell expansion and preparation have been established.
- ✓ Common media also preserves the function of immune cells.
- ✓ Read outs for individual organ models and immune cell monitoring have been established.

Required Materials & Equipment

Provided by InSphero AG

- Akura™ Twin Microplates with Lids
- Akura™ Flow All-in-One Tilter

Optional:

- Assay-ready 3D InSight™ Microtissues in 96 or 384 format (liver, tumor)
- 3D InSight™ Culturing media
- Akura™ 96 Microplates
- Akura™ 384 Microplates

Provided by Customer

- Pipette and tips (e.g., INTEGRA 8-channel, cat. No. 4626 or INTEGRA 12-channel, cat. No. 4633 or INTEGRA 16-channel, cat No. 4642)
- Milli-Q water
- Fetal Calf Serum
- Laminar flow bench
- Plate centrifuge (e.g. Thermo Scientific™ Heraeus™ Multifuge™ X3R; or Sigma 2-6 compact centrifuge)
- Microwave or 37°C water bath (98°F)
- Inverted microscope
- Humidified 5% CO_2 incubator 37°C (98°F)

Optional:

- Integra VIAFLO 384, 125-µl-tip head (cat. No. 6031)
- Opentrons OT-2 liquid handler

Basic microplate handling steps

The Akura™ Twin Microplate has a unique well design that protects spheroids and cells from accidental aspiration during routine pipetting tasks and ensures accurate dilutions for compound dosing. It is important to understand how to orient the plate and pipette tips to take advantage of the special features of the plate (Figure 3). Basic pipetting steps in the Akura™ Twin Microplate are similar to the Akura™ 384 Spheroid Microplate and described in the following paragraphs. However, due to the interconnected wells, some pipetting steps are different.

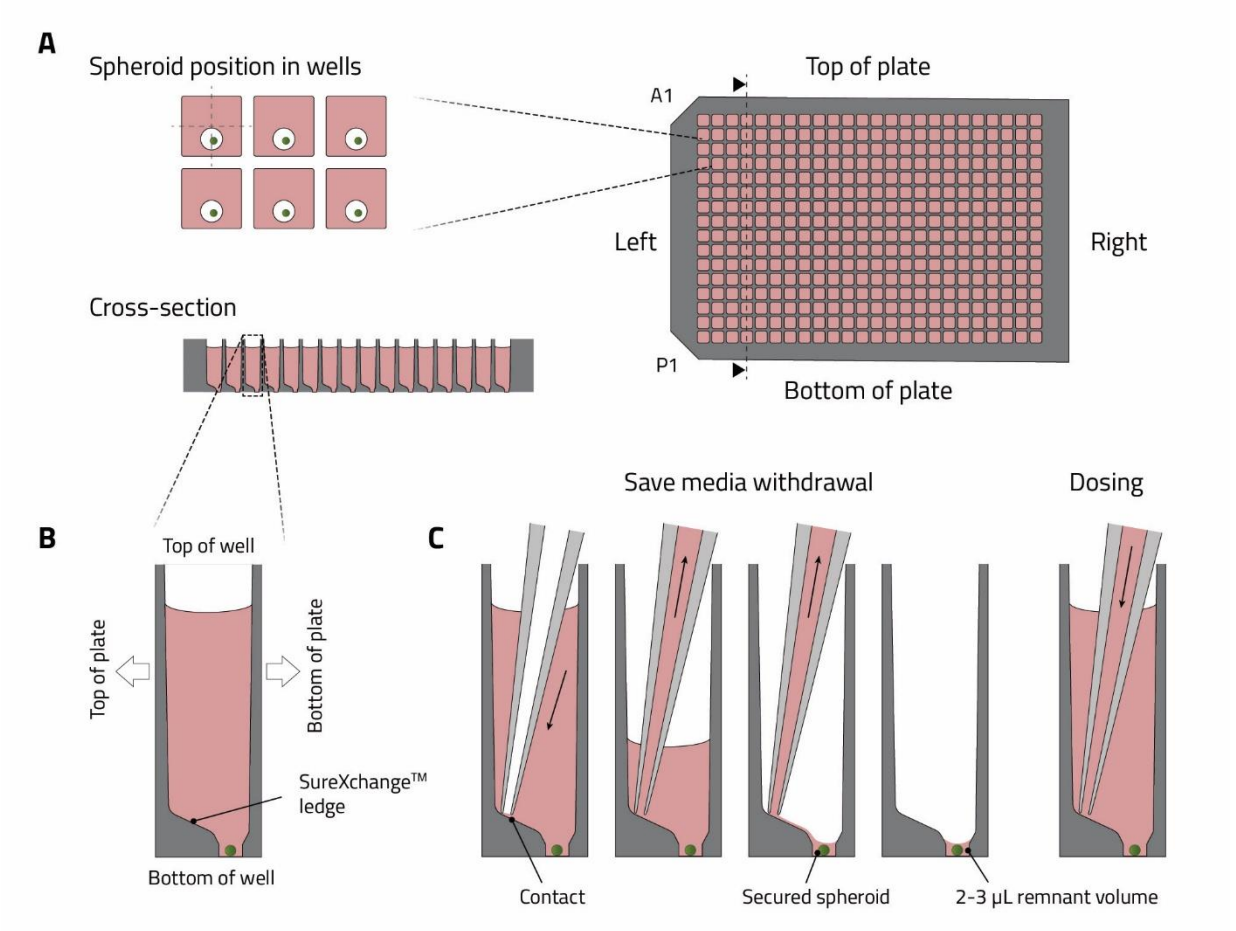


Figure 3: A) The plate layout is 16 rows by 24 columns with well markings on the left side from top to bottom (A-P) and on the top from left to right (1-24). Plate orientation notches are located at the A1 and P1 well corners. Spheroids are secured at the bottom of the plate wells and are positioned centrally in the lower vertical half of the well. B) A side view of the well design illustrates the position of the SureXchange™ ledge that protects the spheroid from accidental harvesting. C) The well design facilitates safe media withdrawal, consistent media remnant volume, and accurate compound dosing. (Pipette angle for right-handed plate orientation shown.)

Plate orientation for manual pipetting tasks

Important: The off-set position of the SureXchange™ ledge and spheroid cavity necessitate correct orientation of the plate and positioning of pipette tips. To avoid pipetting errors, use the same plate orientation for the duration of a given experiment.

For right-handed operators: Turn the Akura™ Twin Microplate 90° to the left, such that the A1 orientation notch is on the left-hand side and the P1 orientation notch is on the right-hand side (Figure 4A).

For left-handed operators: Turn the Akura™ Twin Microplate 90° to the right, such that the A1 orientation notch is on the right-hand side and the P1 orientation notch is on the left-hand side (Figure 4B).

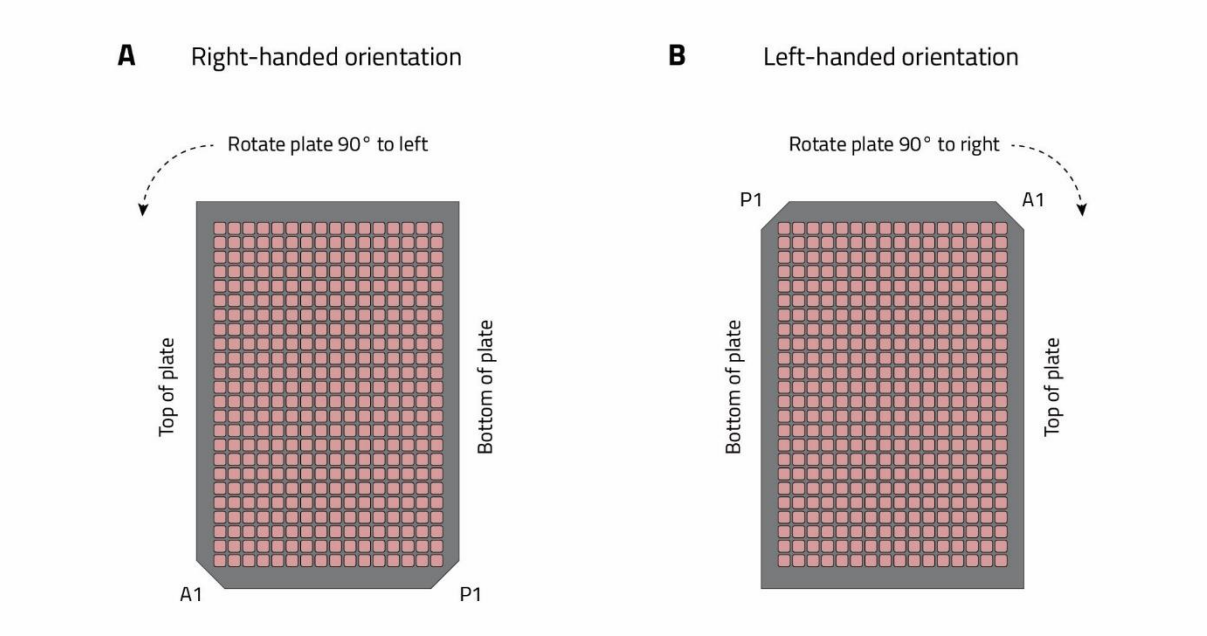


Figure 4: Recommended plate orientation for A.) Right-handed operators and B.) Left-handed operators.

For manual pipetting tasks that do not involve harvesting the spheroid for downstream processing and analysis, always orient the plate (based on operator preference). Then, position the pipette tip against the well wall at an angle towards the top of the plate, and slide it downwards until the SureXchange™ ledge is reached (Figure 3, C). Then, aspirate the complete liquid volume. The spheroid and cells in the cavity and channel are protected and will remain in the well.

Considerations for microscopy

A transparent cyclo-olefin polymer foil (COP) with a thickness of 188 µm covers the complete black plate body at the bottom. Spheroids located in the wells will remain in the wells over the duration of the experiment and are visible in brightfield and fluorescence mode (the transmitted illumination passes the well). Cells (e.g. suspended immune cells) in the microchannels are only visible by illuminating from the bottom with fluorescence microscopy.

The spheroids are within the 1-mm diameter compartment at the bottom of the well. The small diameter compartment ensures quick location of the microtissue and can be used as scale bar for rough size measurement. Due to the special plate wall design, the 1-mm compartment is not in the center of the well. It has an off set of 0.9 mm towards the bottom of the plate. Well-to-well and compartment-to-compartment distance remain the standard of 4.5 mm (Please refer to the Technical Specifications for more details).

The adhesive that connects the COP foil to the black plate body emits autofluorescence in the DAPI channel. This enables precise localization of the channel boundaries during fluorescence microscopy. However, the autofluorescence should be considered when selecting the fluorophores for the experiment.

Generating 3D Spheroids

Important: The Akura™ Twin Microplate is designed for pre-formed spheroids and organoids. Spheroids need to be pre-formed in dedicated plates, preferably the Akura™ 96 or 384 Spheroid Microplates. Please refer to Appendix "Generating 3D Spheroids" for a general protocol.

Setting up Akura™ Twin Experiments (multi-channel pipette)

In this section, the setup of an Akura™ Twin experiment will be explained. All steps are described for multi-channel pipettes. For other pipetting systems, the parameters might differ. For routine work, semi-automated or fully automated protocols are recommended (see separate documents).

Working with multi-channel electronic pipettes

Table 1: Recommendations for culturing spheroids in Akura™ Twin Microplates

Material/Process	Recommendation
Working volume	100 µl / Twin unit
Medium exchanges	Typically, 2-3 times per week
Pipettes	INTEGRA 8-channel pipette (Cat. No. 4623) INTEGRA 12-channel pipette (Cat. No. 3735) or INTEGRA 125 µl 16-channel pipette (Cat. No. 4642)
Aspiration speed	Slow Speed 1
Dispense speed	Slow Speed 1

Note: Depending on the type of the multi-channel pipette used, pipetting orientation and parallel access to the wells are different. For example, if medium needs to be removed from both wells of a Twin unit in parallel, a multi-channel pipette with 4.5 mm distance between pipet tips is necessary (Figure 5).

Figure 5A illustrates the layout of the Akura™ Twin Microplate. Each Twin unit consists of an alpha-well and a beta-well highlighted in different colors. In Figure 5B, examples are shown on how the different pipette types access the wells differently with a respective orientation of the plate. For partial use of the plate, pipettes can be equipped with less tips.

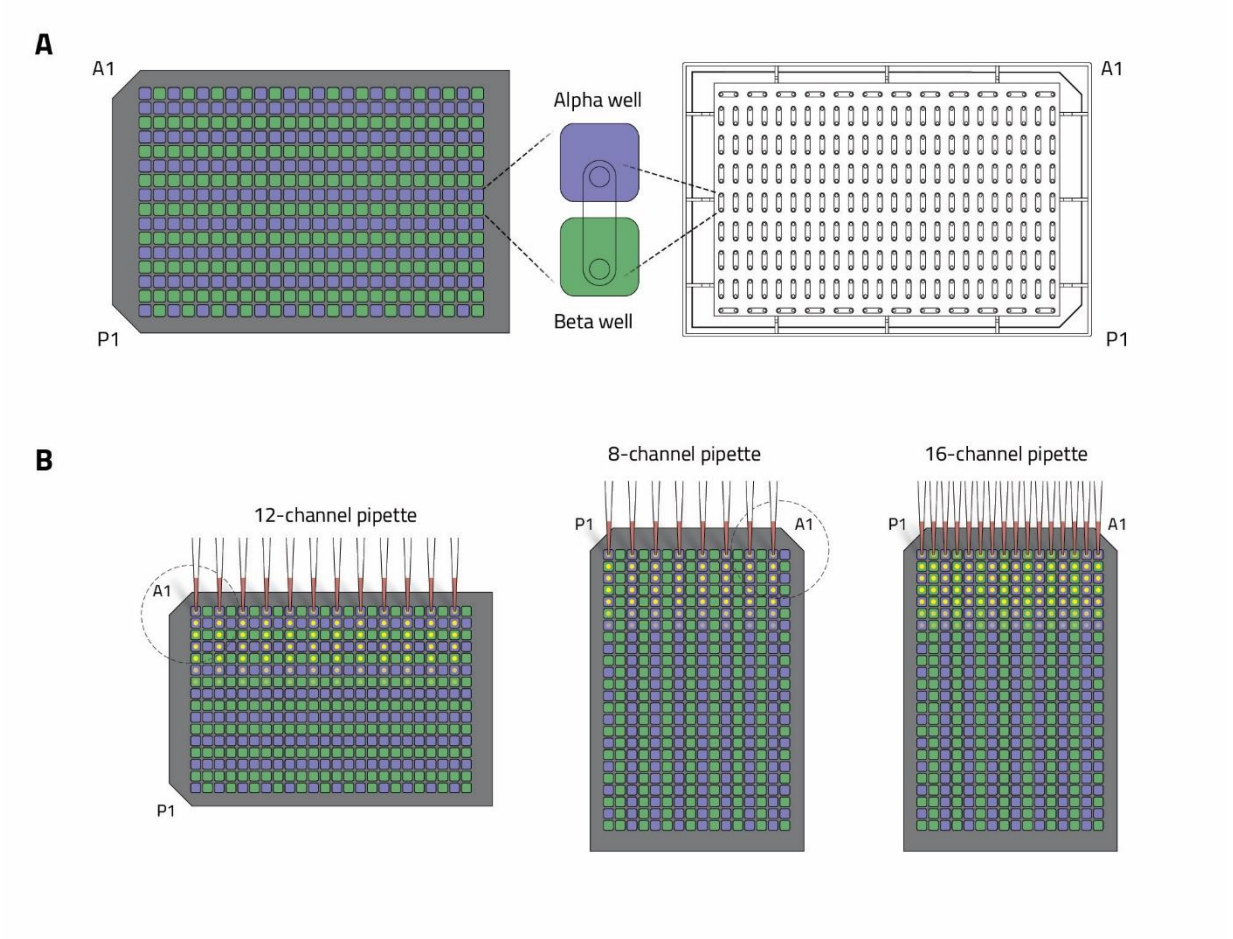


Figure 5: Akura™ Twin Microplate layout and pipetting workflows. The sequence of the first pipette steps is illustrated. A) Each Twin unit consists of an alpha- (blue) and beta-well (green) that are interconnected via a microchannel. Channels are oriented horizontally for row A and P whereas the channels are vertically oriented for rows B to O. B) Row-wise pipetting workflow using a 12-channel pipette. Column-wise pipetting workflow with an 8- or 16-channel pipette.

Part I: Plate preparation (1-2 days before the start of the experiment)

Important: Prior to transferring spheroids into the Akura™ Twin Microplate, initial degassing is highly recommended to prevent inclusion of air bubbles.

1. Pre-warm Milli-Q water to 37 °C (98 °F). You can use a microwave or a water bath.
2. Wipe the Akura™ Twin Microplate bag with 70% EtOH before opening.
3. Carefully open the bag under sterile working conditions e.g. inside a biosafety cabinet and take out the Akura™ Twin Microplate with the lid.

4. Add 90 µl of warm Milli-Q water to every alpha-well (indicated in blue; Figure 5) of the Akura™ Twin unit. Since alpha- and beta-wells are always connected, the liquid will flow from the alpha-well through the channel into the beta-well. Liquid equilibration takes around 3 minutes.

   Note: Either use a 12-channel pipette or a 16-channel pipette

5. Centrifuge the Akura™ Twin Microplate for 2 minutes at 500 RCF and incubate the plate in a humidified CO_2-incubator for **at least 1 day.**

   Note: Air bubbles might appear in the channel. The next section describes the procedure on how to remove air bubbles from the system.

   Note: Do not touch the bottom of the well with the pipette tips as it consists of a 188 µm thin COP-membrane.

Part II: Akura™ Twin loading (start date of the experiment)

1. Pre-warm cell culturing medium to 37 °C (98°F).

2. If air bubbles have appeared, centrifuge the Akura™ Twin Microplate for 2 minutes at 500 RCF. Optional: Turn Akura™ Twin Microplate by 180° and centrifuge again for 2 minutes at 500 RCF.

3. Aspirate Milli-Q water row-wise e.g. with a 12-channel pipette. Position the pipette tip against the well wall of the alpha-well of the Twin unit by starting in B1 (Figure 6, left) and slide it downwards until the SureXchange™ ledge is reached (Figure 3C). Aspirate ≥ 60 µl from every alpha-well in the first row (indicated in blue, Figure 6 left) at low to medium pipetting speed.

4. Subsequently, within the next 5 seconds aspirate again ≥ 60 µl from the adjacent beta-wells C1 (indicated in green, Figure 6 left) of the Twin unit by placing the tip of a 12-channel pipette on to the ledge of the beta well.

   Note: Step 3 & 4 can be repeated if needed.
   Note: A small amount of water (< 10-12 µl) remains in the bottom of the units and in the channel.
   Note: Milli-Q water can also be removed column-wise with a 16-channel pipette (Figure 6, right).

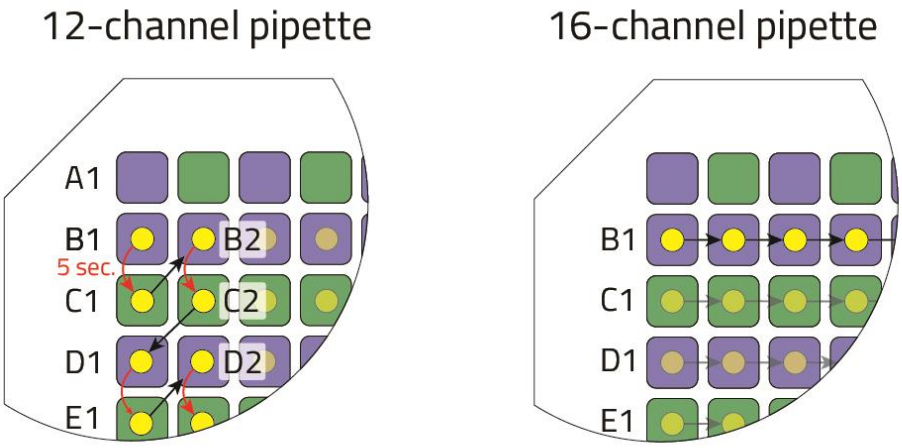


Figure 6: Illustration of pipetting workflow for liquid aspiration in Akura™ Twin Microplate. A) Row-wise with a 12-channel pipette in two successive steps. B) Column-wise with a 16-channel pipette.

5. Flush microchannel by adding 100 µl of cell culturing medium to every alpha well of the Akura™ Twin Microplate unit (indicated in blue, Figure 5) and let liquid levels equalize.

   Note: Liquid equalization takes around 2-3 minutes.

6. Centrifuge the Akura™ Twin Microplate for 2 minutes at 500 RCF.

7. Exchange medium by placing the tip of a multichannel pipette on to the ledge of the alpha well. Aspirate ≥ 60 µl from every alpha well (indicated in blue, Figure 5) at low to medium pipetting speed.

8. In a second step, aspirate again ≥ 60 µl from every beta well by placing the tip of multichannel pipette on to the ledge of the beta well (indicated in green, Figure 5) at low to medium pipetting speed.

9. Before spheroid transfer, add 20 – 30 µl warm culture medium to all wells.

Spheroid transfer

The Akura™ Twin Microplate is an assay plate. Due to the construction of the plate (interconnected wells) it is not possible to aggregate spheroids in the Akura™ Twin Microplate. We highly recommend using the Akura™ 96 or 384 Spheroid Microplates for aggregation and pre-treatment of the spheroids

before the transfer into Akura™ Twin Microplate (Figure 7). For detailed spheroids aggregation protocols please see appendix "Generating 3D Spheroids".

Important: It is recommended to perform a medium exchange before spheroid transfer. A medium exchange moves spheroids gently and facilitates spheroid aspiration.

Note: To avoid evaporation edge effects, it is recommended to use edge wells (A1-A24, A1-P1, P1-P24, A24-P24) as evaporation barrier by filling the wells with PBS or Milli-Q water.

Semi-automated and automated spheroid transfer

The design of the Akura™ Twin Microplate allows for semi-automated and fully automated spheroid transfer using e.g. Opentrons OT-2 robot. Optimized protocol setups will be shared upon request.

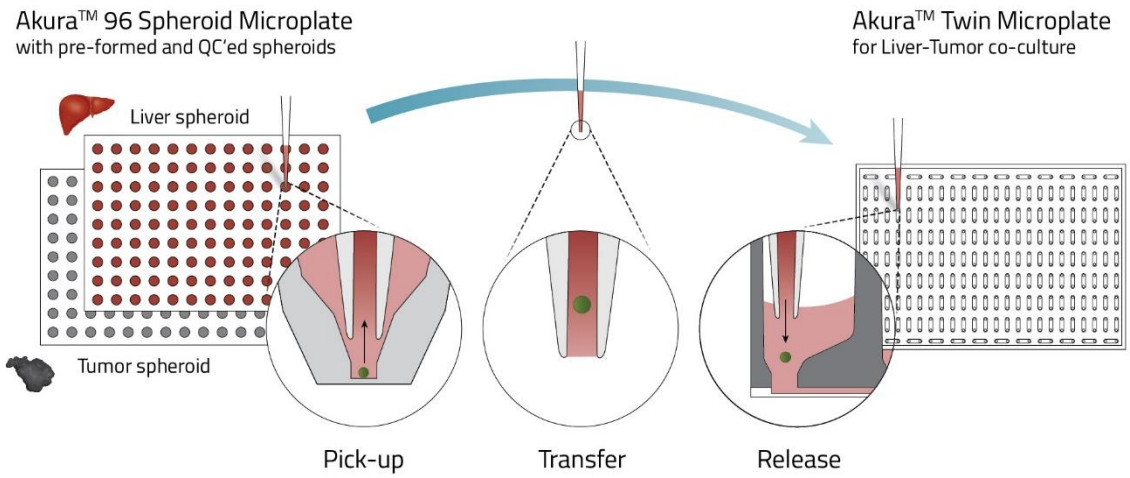


Figure 7: Akura™ Twin Microplate Spheroid transfer workflow. Off-chip production and on-chip integration. Akura 96 Spheroid Microplate with pre-formed and QC'ed spheroids were picked-up in 40 µl and released into one well of a Twin unit. Transfer is illustrated for manual single-channel pipette but can be similar performed with a multi-channel pipette, semi-automated with INTEGRA Viaflo 96/384 systems or automated with a robotic system.

Spheroid transfer from Akura™ Spheroid Microplates using a single channel pipette:

1. Pre-wet the 100-200 µl tip with 100% serum to prevent spheroids from sticking to the inside walls of the plastic tip.

   Note: Alternatively, tip can be coated with cell culture medium containing FCS / BSA.

2. Gently immerse the pipette, holding a 100-200 µl tip, along the inside wall towards the bottom of the well until feeling a slight resistance. The pipette tip orifice is now positioned slightly above the spheroid on the well bottom.
3. Collect the spheroid by aspirating 40 µl of medium at medium to high speed. Depending on the spheroid and pipette type in use, different volumes, speeds, and repetitions are required to successfully aspirate and eventually transfer the spheroid. Avoid aspiration of air bubbles to prevent spheroid loss in the pipette tip.
4. Visually check if the spheroid was aspirated. Spheroids are visible as small white dots sinking down by gravity in the tip.

   Note: Keep the pipette as vertical as possible
5. Gently dispense spheroid into the desired well in the Akura™ Twin Microplate.
6. Repeat steps 2-6 until all spheroids have been transferred.
7. OPTIONAL: Centrifuge the Akura™ Twin Microplate for 1 minute at 250 RCF to bring the spheroids in the observation window of each well.
8. Inspect the spheroid transfer by light microscope or plate scanner.
9. Exchange transferred culture medium by placing the tip of a multichannel pipette on to the ledge of the alpha well. Aspirate ≥ 60 µl from every alpha well (indicated in blue, Figure 5) at low to medium pipetting speed.
10. In a second step, aspirate again ≥ 60 µl from every beta well by placing the tip of multichannel pipette on to the ledge of the beta well (indicated in green, Figure 5) at low to medium pipetting speed.
11. Add 100 µl of fresh medium to the alpha-well of the Twin unit and let liquid levels equalize between Twin wells.

Suspension cell loading (e.g. PBMCs)

The Akura™ Twin Microplate allows for co-culturing spheroids with suspension cells e.g. PBMCs. Cells can be loaded and distributed either evenly between both wells of the Twin unit (Figure 8 A) or exclusively into one of the wells (Figure 8 B).

Note: It is recommended to use fluorescently labelled suspension cells to observe potential adhesion and infiltration into the spheroids and to localize suspension cells in the Twin unit. The CellTrace™ (ThermoFisher Scientific) family of dyes can be utilized for that purpose.

Distributed loading of suspension cells to the Twin unit (Figure 8, A):

1. Prepare suspension of fluorescently labelled cells of interest in desired medium and number per ml.
2. Before suspension cell loading, aspirate medium by placing the tip of a multichannel pipette on to the ledge of the alpha well of the Twin unit. Aspirate ≥ 60 µl from every alpha well (indicated in blue, Figure 5) at low to medium pipetting speed.
3. In a second step, aspirate again ≥ 60 µl from every beta well of the Twin unit by placing the tip of a multichannel pipette on to the ledge of the beta well (indicated in green, Figure 5) at low to medium pipetting speed.
4. Add 100 µl of cell suspension to the alpha well of every Twin unit. Let the level of medium equalize between the units. Liquid equalization takes around 2-3 minutes.
5. Let suspension cells sediment for 30 minutes in the incubator before placing on the Programmable All-in-One Tilter.

Loading of suspension cells to only one well of the Twin unit (Figure 8, B):

1. Prepare suspension of fluorescently labelled cells of interest in desired medium and cell density.

   Note: The cell density needs to be adjusted (20X) to reach the correct final density in the Twin unit.
2. Before suspension cell loading, aspirate medium by placing the tip of a multichannel pipette on to the ledge of the alpha well of the Twin unit. Aspirate ≥ 60 µl from every alpha well (indicated in blue, Figure 5) at low to medium pipetting speed.
3. In a second step, aspirate again ≥ 60 µl from every beta well of the Twin unit by placing the tip of a multichannel pipette on to the ledge of the beta well (indicated in green, Figure 5) at low to medium pipetting speed.

   Note: Steps 4 and 5 must be carried out immediately one after the other.
4. Add 95 µl of fresh medium to the beta well of the Twin unit.
5. Subsequently, add 5 µl of cell suspension to the alpha well of the Twin unit, while the level of medium equalizes between the units.
6. Let suspension cells sediment for 30 minutes in the incubator at 37°C before placing it on the Akura™ Programmable All-in-One Tilter.

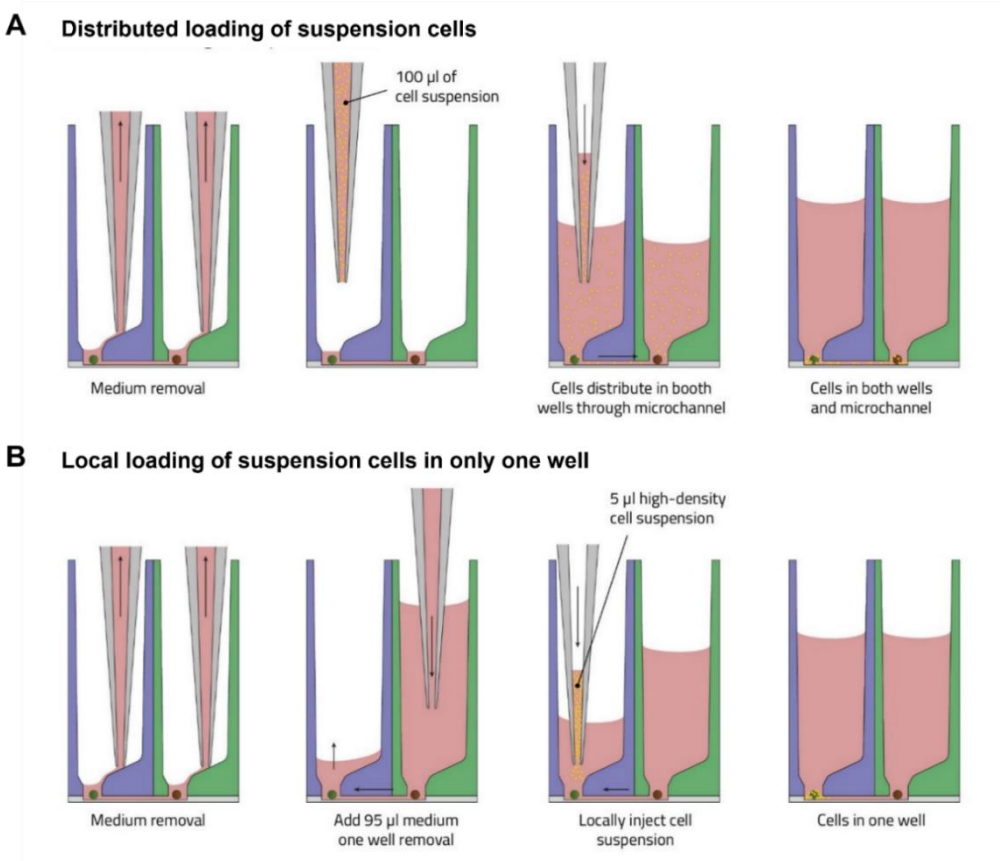


Figure 8: Loading cell suspension into Akura™ Twin Microplate. Alpha-wells are indicated in blue and beta-wells in green. A) Procedure to achieve an equal distribution of suspension cells e.g. PBMCs in both wells of the Twin unit. B) Suspensions cells are loaded in a small volume of 5 µl into one well of the Twin unit to confine suspension cells on one side only.

Medium Exchange in Akura™ Twin (multi-channel pipette)

Medium exchange with multi-channel electronic pipettes

Cultivating spheroids typically requires 2-3 medium exchanges per week. Depending on your experimental design (presence or absence of suspension cells), we recommend two different pipetting workflows:

- In the **presence of suspension cells**, the medium needs to be **aspirated simultaneously** from alpha and beta well of each Twin unit. Otherwise, the liquid in the other well will always refill the just emptied well through the connecting microchannel. Eventually, equally distributed PBMCs will be moved by the flow and accumulate in only one well. (please follow the steps "column-wise medium exchange").

  Note: Keep Akura™ Twin Microplate for 5-15 minutes (depending on tilting angle) in horizontal position after removing the microplate from the tilter to allow sedimentation of suspension cells to the bottom. This step ensures that suspension cells will not be aspirated.

- In the **absence** of suspension cells, the medium exchange can be performed either column- or row-wise (please follow the steps "column-wise medium exchange" or "row-wise medium exchange).

Performing column-wise medium exchange

1. Orient the Akura™ Twin Microplate based on operator preference (Figure 4).
2. If using an electronic multi-channel pipette (recommended), set it to a slow speed (e.g. Speed 1).
3. Aspirate carefully ≥ 60 µl medium **simultaneously** by placing the tip of a 16-multichannel pipette on to the ledge of the alpha and beta well of the Twin unit. This will lead to an almost complete removal of the medium.

   Note: Medium needs to be removed from alpha- and beta-wells simultaneously, which is achieved with a 16-multichannel pipette. Otherwise, the liquid in the other well will always refill the just emptied well through the connecting microchannel and eventually equally distributed PBMCs will be moved by the flow and accumulate in only one well.

   Note: Aspiration volume from alpha and beta well is set to ≥ 60 µl each to perform complete medium removal if total working volume in Twin unit was set to 100 µl.

   Note: A small amount of medium (< 10-12 µl) remains in the bottom of the units and in the channel.

4. Add 50 µl of pre-warmed medium simultaneously by placing the 16-channel pipette on to the ledge of the alpha and beta well of the Twin unit and gently dispense at a slow pipetting speed.
5. Optional: For a more thorough medium exchange, repeat steps 3 and 4.

Performing row-wise medium exchange

1. Orient the Akura™ Twin Microplate based on operator preference (Figure 4).
2. If using an electronic multi-channel pipette (recommended), set it to a slow speed (e.g. Speed 1).
3. Carefully aspirate medium row-wise by placing the tip of a multi-channel pipette on to the ledge of the alpha well of the Twin unit. Aspirate ≥ 60 µl from every alpha-well (indicated in blue, Figure 5).
4. Subsequently, within the next 5 seconds, aspirate again ≥ 60 µl from the connected beta well (indicated in green) of the Twin unit by placing the tip of a multi-channel pipette on to the ledge of the beta well (Figure 5 and Figure 6).

   Note: Aspiration volume from alpha and beta well is set to ≥ 60 µl each to achieve complete medium removal if total working volume in Twin unit was set to 100 µl.

   Note: A small amount of water (< 10-12 µl) remains in the bottom of the units and in the channel.
5. Add 100 µl of fresh cell culturing medium to every alpha-well of the Akura™ Twin unit and let liquid levels equalize. Liquid equalization takes around 2-3 minutes.
6. Optional: For a more thorough medium exchange, repeat steps 3 to 5.

Long-term culturing

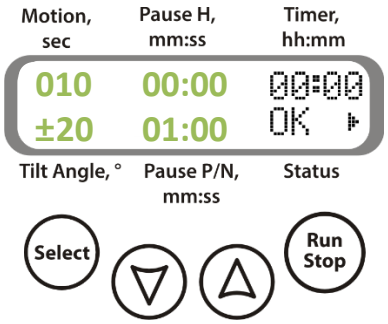
In this section microtissue culturing with and without suspension cells are described:

1. Wipe Akura™ All-in-One Tilter with 70% ethanol and place it into an incubator. Note: Fix the cable with a tape to the incubator box.

2. Program tilting sequence using the built-in display and buttons (Please refer to InSphero Akura™ Programmable All-in-One Tilter Manual for more details).

   Note: For Akura™ Twin Microplates loaded with 100 µl of medium per Twin unit without suspension cells, the following settings are recommended:

   - Tilt angle: ± 20°
   - Motion time: 10 s
   - Pause at (P/N = ± 20°) for 1 min
   - Pause at (H = ± 0°) for 0 min
   - Timer: 0 s to run without any time limit



   Note: For reliable exchange of suspension cells between alpha and beta well, the following settings are recommended:

   - Tilt angle: ± 40°
   - Motion time: 5 s
   - Pause at (P/N = ± 20°) for 1 min
   - Pause at (H = ± 0°) for 0 min
   - Timer: 0 s to run without any time limit

   Note: Alternative tilting settings should be tested beforehand depending on the type of suspension cells. An increase in the total cell culture medium, for example, requires the reduction of the tilting angle to avoid spillover. A lower cell culture medium volume may increase evaporation effects.

3. Place the Akura™ Twin Microplate on one of the platforms of the 4-plate rack and position it properly in the cavity such that it is well secured.

   Note: Each Akura™ Twin Microplate should be incubated on the same position with the same orientation throughout the experiment – also after medium exchange. Well A1 is facing towards the back side of the incubator. Up to 4 Akura™ Twin Microplates can be placed on one Akura™ Flow All-in-One Tilter. For tilt angles above 20° fixation of the plate to the plate rack of the tilter using adhesive tape is recommended.

4. Start the tilting program to induce liquid exchange between the Twin wells.

5. Medium exchange should be performed every 2-3 days. If longer periods are needed, please check evaporation.

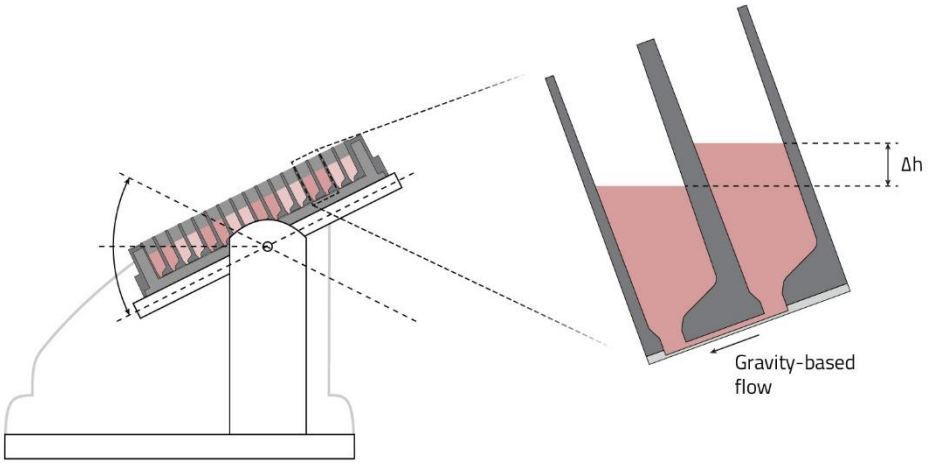


Figure 9: Illustrating the Akura™ Twin Microplate on the tilter. Gravity-based flow is induced by tilting back and forth. Gravity-driven flow relies on hydrostatic pressure caused by height differences between two wells. By changing the tilting angle, the flow rate can be modulated.

Analysis and Assays in Akura™ Twin Microplate

The Akura™ Twin Microplate format is compatible with a broad variety of biochemical methods and allows for spectrometric measurements with a multi-well plate reader or for visual inspection of spheroids by an inverted microscope.

Fluorescent/luminescent multi-well plate reader compatibility

Growth changes and profiles in tumor spheroids expressing GFP/RFP can easily be analyzed using fluorescent plate readers, as the signal intensity is stronger than with monolayer cultured cells.

Automated imaging

The Akura™ Twin Microplate is ideal for use with automated imaging equipment, such as the SCREEN Cell3iMager and automated microscopes, as the Akura™ Twin Microplate features a continuous 188 µm COP (cyclo-olefin polymer) bottom resulting in enhanced imaging quality. The black-walled body minimizes fluorescent crosstalk between wells and the 1.0 mm diameter optically clear base of each well will be positioned exactly in the field of view.

Note: The adhesive that connects the COP foil to the black plate body emits autofluorescence in the DAPI channel. This enables precise localization of the channel boundaries during fluorescence microscopy and allows for automated channel recognition by an image analysis software. However, autofluorescence should be considered when selecting the fluorophores for the experiment.

The Akura™ Twin Microplate is compatible with high-content imaging instruments such as Opera, Operetta (Perkin Elmer), CQ1, CellVoyager CV 8000 (Yokogawa) or ImageXpress (Molecular Devices).

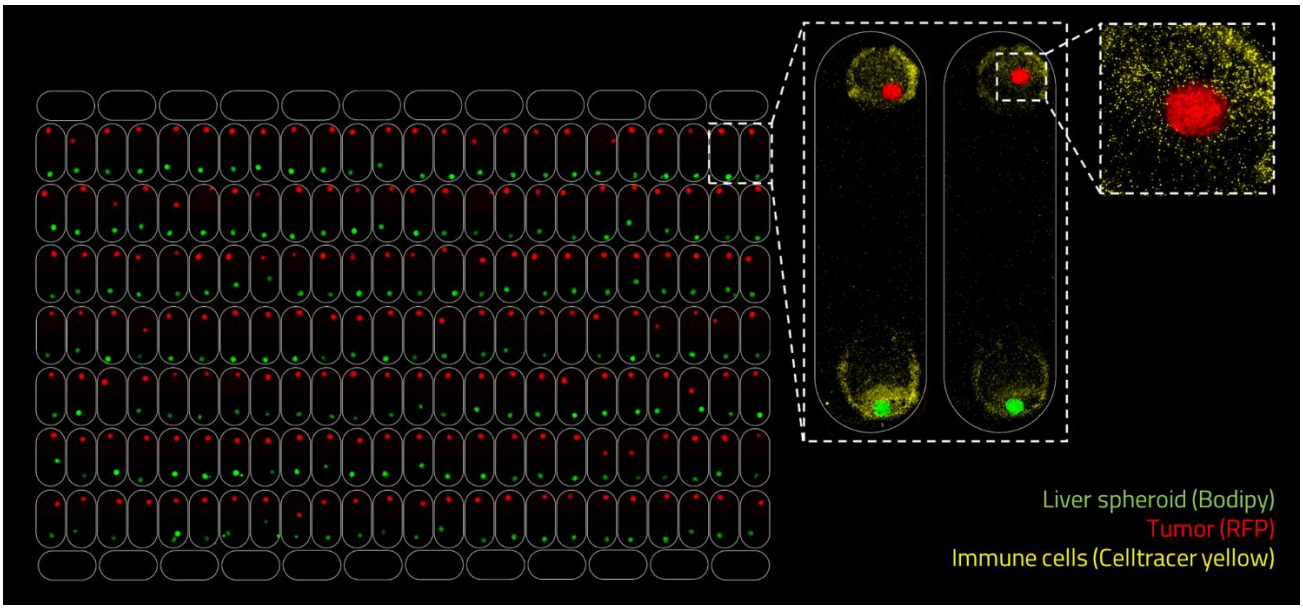


Figure 10: Images of 3D InSight™ hLiMT and 3D InSight™ hTuMT co-cultures in the presence of PBMCs taken with Yokogawa CQ1 (picture selection) and Leica DMi8 microscope (whole Akura™ Twin Microplate).

Spheroid Collection and Transfer

Spheroid collection

The special coating of the Akura™ Twin Microplate minimizes the adherence of the spheroids to the bottom of the well. This facilitates the collection of spheroids for transfer into another plate format or for further processing, such as embedding for histological analysis. There are several different methods for collecting and transferring spheroids.

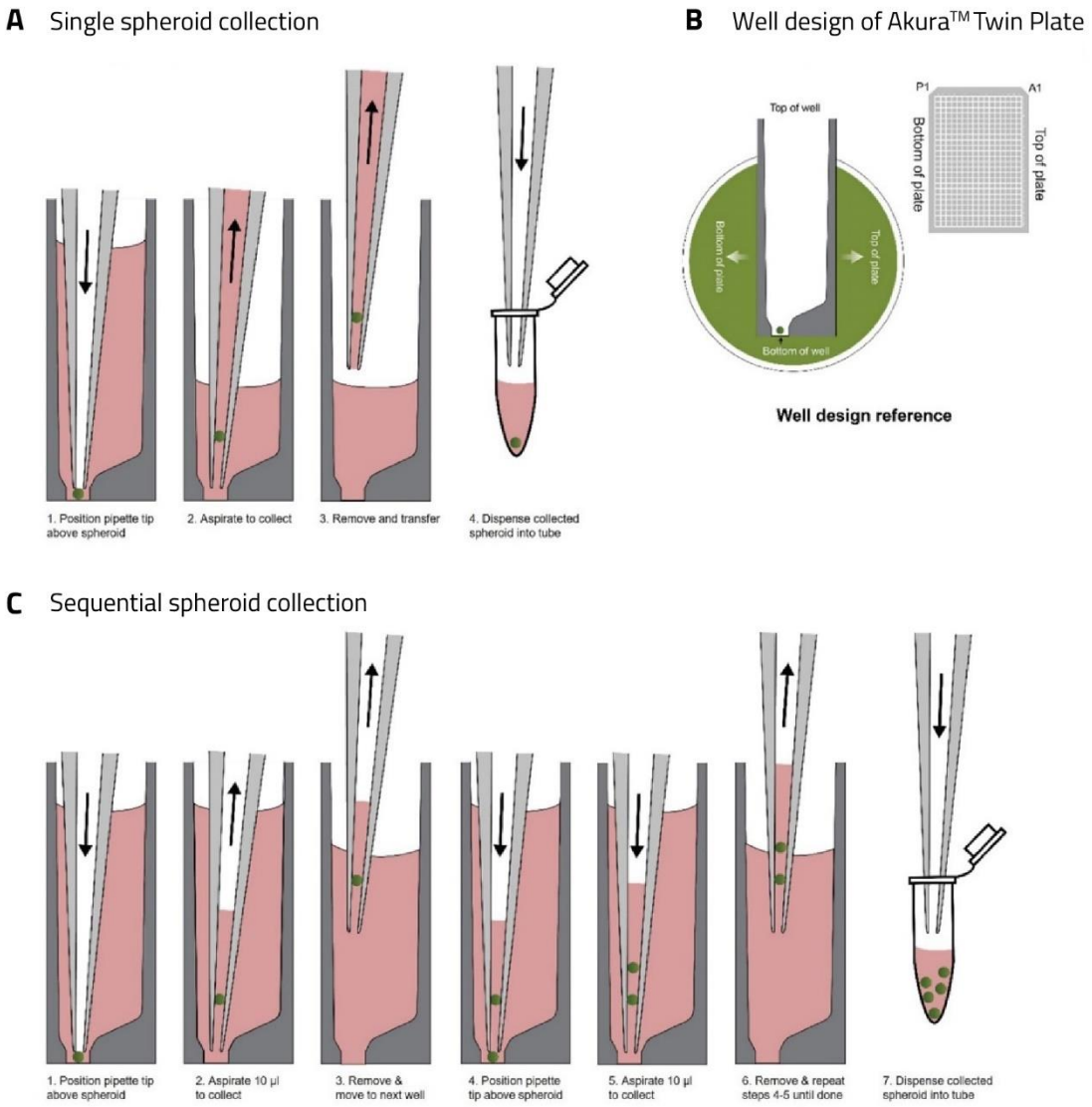


Figure 11: A.) Pipette positioning and steps for collecting and transferring a single spheroid from Akura™ Twin Microplates for downstream processing. B.) Reference of well design in relation to top and bottom of plate. C.) Steps for sequential spheroid collection and transfer.

Single spheroid collection and transfer using a single channel pipette:

1. Pre-wet the tip with 100% serum or culture medium containing serum to prevent spheroids from sticking to the inside walls of the plastic tip.
2. Gently immerse the pipette tip into the well until feeling a slight resistance (e.g. 1250 µl tip, Greiner Bio-One Sapphire, Cat. No. 750 265) and position the tip orifice above the spheroid on the well bottom. Use of this tip guards against accidentally squeezing spheroids during collection because the tip diameter exceeds the size of the well bottom.
3. Collect the spheroid by aspirating 40 µl of medium at medium speed. Depending on the spheroid and pipette type in use, different volumes, speeds, and repetitions are required to successfully aspirate and eventually transfer the spheroid. Avoid aspiration of air bubbles to prevent spheroid loss in the pipette tip.
4. Gently dispense spheroid into another vessel or plate.

Note: When using a recipient plate that is already pre-filled with medium, there is no need for a dispensing step. Simply immerse the pipette tip containing the spheroid into the medium in an upright position. Allow 30-60 seconds for the spheroid to settle at the bottom of the well by gravity.

Sequential spheroid collection and transfer using a multi-channel pipette:

1. Pre-wet the tip with 100% serum or culture medium containing serum to prevent spheroids from sticking to the inside walls of the plastic tip.
2. Set your multi-channel pipette to "variable/multi aspirate" mode, 8-12 aspiration steps of 10 µl, speed 5-10. Load one 300 µl tip, with or without filters, depending on downstream activity (e.g., sterile: INTEGRA, Cat. No. 4435; unsterile: INTEGRA, Cat. No. 6443).
3. Gently immerse the multi-channel pipette, holding one 300 µl tip, along the inside of the well wall until a slight resistance is felt. This positions the pipette tip orifice slightly above the spheroid on the well bottom.

   Note: The diameter of the 300 µl tip does not exceed the size of the well bottom, so use caution not to squeeze the spheroid or pierce the well bottom accidentally.
4. Collect the spheroid by aspirating 10 µl of medium with speed 5-10. Repeat the aspiration step for the same spheroid (to increase collection efficiency) or remove pipette from well and continue to the next well/spheroid. Collect additional spheroids by aspirating another 10 µl. Execute all the predefined aspiration steps.

   Note: The number of aspiration steps may vary depending on the number of spheroids to be collected. Different volumes, speeds, and repetitions may be required to successfully collect different types of spheroids.
5. Gently dispense all collected spheroids into collection vessel.

APPENDIX

Generating 3D Spheroids

Important: The Akura™ Twin Microplate is designed for pre-formed spheroids and organoids. Spheroids need to be pre-formed in dedicated plates, preferably the Akura™ 96 or 384 Spheroid Microplates (Appendix "Generating 3D Spheroids").

Generating 3D spheroids in the Akura™ Spheroid Microplates is a straightforward process, but one that must be optimized for each cell type. Here, the process is only briefly described for the Akura™ 384 Microplate. Please refer to dedicated application notes and plate manuals or contact InSphero AG. Cell type, growth medium, and intended downstream applications will impact the starting density and desired culture volume. Optimization is recommended for each cell type and application.

Additional Materials Required

1. Mammalian cells (primary or cell line) of interest
2. Your specific maintenance medium
3. Inverted microscope with a 5x/10x objective
4. Cell counter, e.g. Neubauer chamber
5. Single channel pipette, multichannel pipette (e.g., INTEGRA 8-channel, cat. No. 4626 or INTEGRA 12-channel, cat. No. 4633), or INTEGRA Biosciences VIAFLO 96/384 system (recommended)
6. Medium reservoir for multichannel pipettes
7. Microplate centrifuge
8. Humidified 5% CO_2 incubator 37 °C.

Preparation

1. Prior to seeding, pre-warm the cell maintenance medium to 37 °C.
2. Wipe the Akura™ 384 Microplate bag with 70% EtOH before opening.
3. Carefully open the bag under sterile working conditions e.g. inside a biosafety cabinet and take out the Akura™ 384 Microplate assembly.

Pre-wetting

Important: Pre-wetting the wells of the Akura™ 384 plate according to the procedure below is highly recommended to prevent inclusion of air bubbles. Perform all following steps under sterile conditions:

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™ Microplate 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™ Microplate from the incubator, centrifuge the Akura™ Microplate 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 compartment.

Cell seeding

1. Prepare a single-cell suspension of your preferred cell type or cell mixture according to your standard protocol.
2. Count the cells using a Neubauer chamber or cell counting instrument to determine the starting cell concentration.
3. Prepare the final cell suspension for seeding, using a final volume per well of 50 µl. Recommended cell concentrations: For long-term growth profiling start with low cell numbers (250–500 cells per well). If non-proliferating cells or rapid production of larger spheroids/organoids are required, start with 2500–25,000 cells per 50 µl.

Important: To generate spheroids with uniform size and cell composition, it is essential to assure a homogeneous distribution of the cell suspension by gently pipetting up and down prior to seeding into the Akura™ 384 Microplate.

4. Gently (≤10 µl/sec) add 50 µl of the cell suspension to the Akura™ 384 Microplate by placing the pipette tips far into the wells (avoid touching the well bottom)

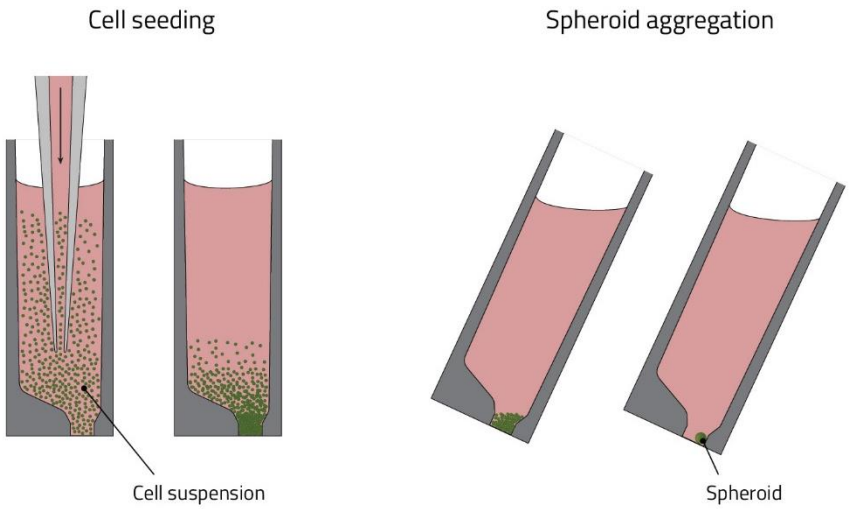


Figure 12: Cross-section of one well illustrating the cell seeding and spheroid aggregation process. The risk of multiple spheroid clusters is reduced if the plates are tilted during spheroid aggregation to concentrate cells in one edge and increase cell-to-cell contact.

Cell sedimentation and spheroid maturation

Following seeding, it is recommended (but optional) to briefly centrifuge the plate to remove any air bubbles, and to force cells to the bottom of the well to promote aggregation and spheroid formation.

1. Place the lid on the Akura™ 384 Microplate and spin in a microplate centrifuge for 2 minutes at 250 RCF.
2. Following centrifugation, remove the plate and incubate the plate in a humidified 5% CO_2 incubator at 37°C for 2-5 days, checking daily to observe spheroid maturation and exchanging medium if necessary.
3. Tilt the plate in the incubator to approximately 30° by leaning it against another plate or use the Akura™ Tilting Stand (InSphero AG, CS-AG11) to improve the maturation process.

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