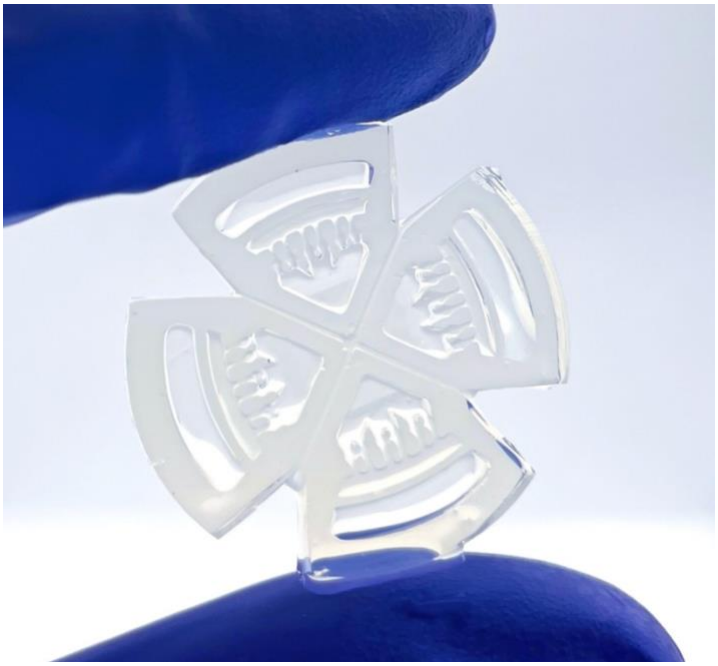
APRICELL BIOTECHNOLOGY

3-in-1 Plate

NEXT GEN TISSUE CULTURE



User Protocol

Invasion Protocol

Materials Included

- Six transparent hydrogel inserts.
- One standard EPS six-well plate

Shipping & Storage

The inserts are shipped in 6 well plate and sealed sterile bags. The plates can be stored at 4°C temperature for up to 9 months from the date of production.

Important Starting Notes

1. The plates are shipped in a sterilized condition**. However, we recommend putting the plates under the UV light of the biosafety cabinet for 15 min before usage.**
2. To avoid contamination, only manipulate the plates and inserts using aseptic techniques in a laminar flow hood.
3. If condensation forms on the plates, gently wipe with an aseptic wiper.
4. Each insert is covered with a small amount of PBS and Pen-Strep. Aspirate all liquid from each insert before beginning your experiment.
5. When placing the plate in the incubator, gently add 500 µL of PBS or culture media to the center of each **unused** insert to prevent it from drying out.
6. The inserts have been inserted at the bottom of each well for optimal imaging conditions. Removing the inserts and placing them in a new 6-well plate will reduce image quality.
7. Refer to the **3-in-1 Plate Data Sheet** for specific inquiries.
8. **This plate was optimized** for making tumor spheroids from various types of mesenchymal and epithelial cells. The protocol may need adjustments to satisfy your specific needs. Each insert contains four separate seeding compartments suitable for performing four different experimental conditions. The number of cells per seeding can be adjusted from 50K to 350K cells according to your desired spheroid size.

Invasion Protocol

1- Cell Seeding & Tumor Spheroid Formation

***The number of cells seeded in each quadrant can range from 100K to 350K cells according to your desired spheroid size. Each seeding produces six spheroids or organoids. This protocol is optimized for cancer cell spheroids ***

1-1- Using an aseptic technique in a laminar flow hood, open the sealed bag and add 25 µL of culture media in the Cell & ECM Loading Zone (Fig. 1) of each compartment of the insert.

1-2- After 2 minutes, add 50 µL of cell suspension[1] to the Cell & ECM Loading Zone of each compartment of the insert and incubate for 10 minutes under cell culture conditions to let the cells sediment into the microwells[2]. Make sure to deposit the cell suspension close to the gel and drop-by- drop to avoid bubbles getting trapped. This process is illustrated in Fig. 2.

1-2-1- **Optional Direct Co-culture**: any types of stromal cells present in the microenvironment can be prepared in a suspension with the tumor cells and can be seeded in the Cell & ECM Loading Zone of each compartment.

1-3- After the cells have settled into the microwells, gently rinse any additional cells stuck in the channels of the Cell/ECM Loading Zone by adding 70-100µL of pre-warmed medium to the Cell/ECM Loading Zone. Then, aspirate that media by placing the pipette tip as close to the microwells as possible. Remove any additional media from the Secondary Reservoir (see Fig. 1). **Repeat the rinsing step if you observe any remaining single cells in the channels of the Cell/ECM Loading Zone.**

1-4- Finally, add 80 µL of pre-warmed fresh culture media to the Media Reservoir and 80 µL to the Cell & ECM Loading Zone of each quadrant of the insert. Incubate under cell culture conditions.

1-5- Monitor spheroid formation and growth daily[3] and change the media regularly[4] until spheroids reach your desired size.

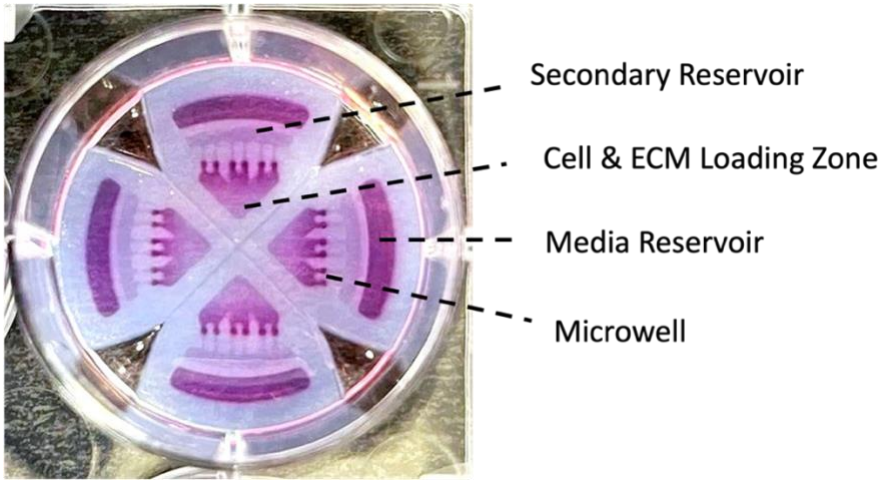


Fig. 1. Labeled Birds-Eye-View of the 3-in-1 Plate

[1] Prepare 50 µL of cell suspension to the desired concentration. The concentration used for a seeding number of 100K cell and 300K cells would be 2 x 10^6 and 6 x 10^6 cell /mL, respectively.

[2] This period of sedimentation may vary according to the cell type. For cells with smaller sizes, you need to incubate for a longer time.

[3] Add fresh media before imaging for optimal image quality.

[4] Refresh media as needed to ensure that media is always covering the spheroids. A variety of factors will affect the rate of media evaporation.

2- Extracellular Matrix Application

2-1- After spheroid formation inside the microwells of each quadrant of the insert, gently aspirate the media from the Media Reservoir[5] and then add 40-60 µL of the desired hydrogel solution to the Cell & ECM Loading Zone [6].

2-1-1 **Optional Co-culture:** any types of normal (stromal) cells present in the microenvironment can be prepared in a suspension with certain concentration and be mixed with the ECM hydrogel solution prior to applying it to the spheroids[7].

2-2- After adding the ECM but before curing[8], leave the plate for 2-5min (resting time)[9] under the laminar flow hood to let the hydrogel solution fill the entire microchannel and microwells of each compartment.

2-3- After cross-linking the hydrogel, gently add 80 µL of pre-warmed fresh culture media to the Media Reservoir and 80 µL to the Cell & ECM Loading Zone of each compartment of the insert. Incubate under cell culture conditions.

2-4- Monitor spheroid formation and invasion daily under the bright field microscope and change the media as needed[4].

2-4-1- Invasion length within the ECM can be monitored over time. After embedding spheroids inside the ECM hydrogels, the cells at the border of the spheroids will start to invade through the connective microchannels, Fig. 3.

2-5- For drug testing, simply mix your compound with media to the desired concentration and add to the Media Reservoir.

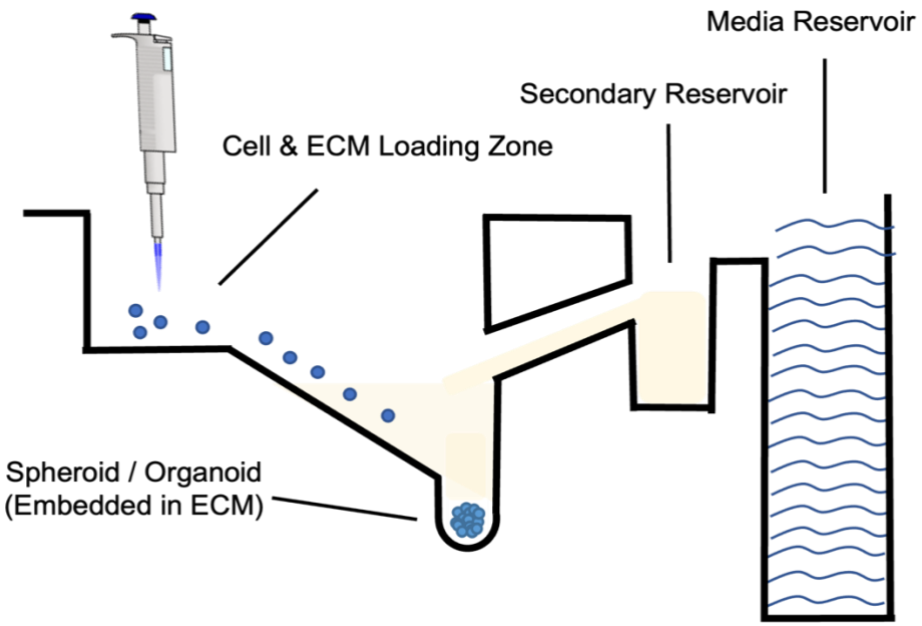


Fig. 2. Schematic cross-section of the 3-in-1 plate with location of cell and ECM loading

[5] After aspirating the media from the Media Reservoir, any additional media left in the microwells can be aspirated from the slope of the Cell & ECM Loading Zone.

[6] The ECM Loading Zone is at the same location as the Cell Loading Zone. It is important to ensure that the hydrogel solution is not excessively viscous to the point that it cannot flow down the microchannels into the microwells.

[7] In co-culture conditions, the concentration of the hydrogel solution should be adjusted with the cell suspension media.

[8] Depending on the cross-linking method of the hydrogel, it can be cured using either temperature or light, etc.

[9] This time may vary based on the viscosity of the hydrogel solution. Solutions with higher viscosity need more resting time.

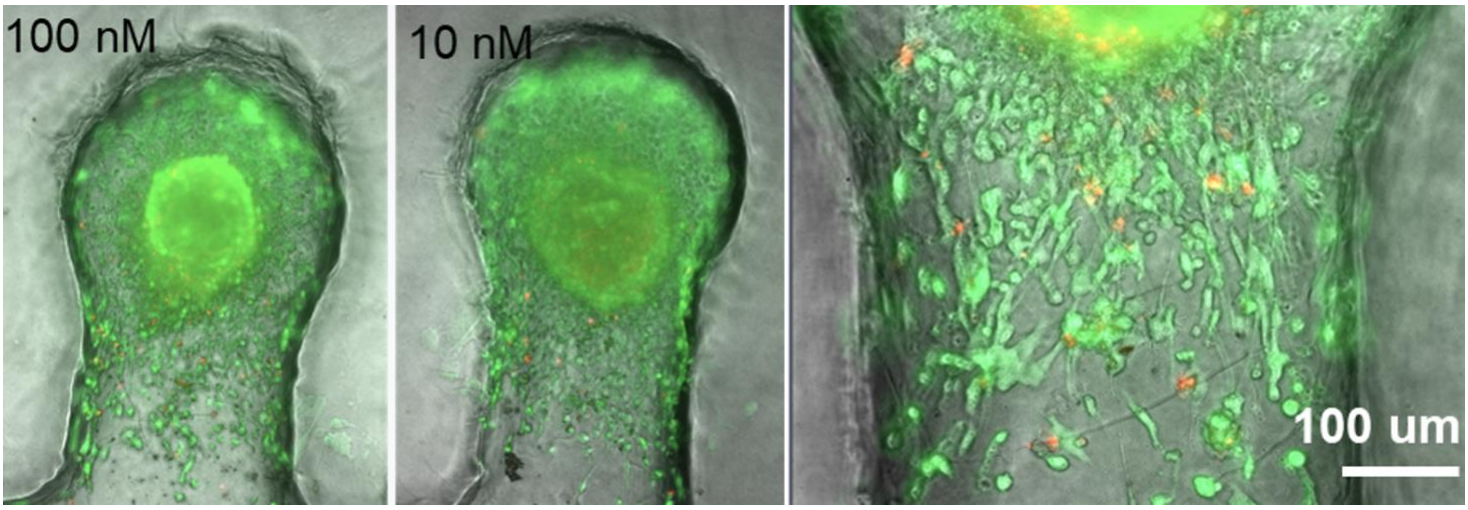


Fig. 3. Fluorescent images of the cancer cells invading into the ECM hydrogel and through the microchannels connected to the spheroid microwells.

3- Imaging & Analysis

- The 3-in-1 Plate is compatible with different imaging modalities including invert, up-right, fluorescent and confocal microscopes.
- For invasion monitoring within the ECM hydrogel, make sure to have sufficient media on top of the gel (in each quadrant of the insert). The insert is in the fixed position inside the 6 well-plate and is compatible with automated image acquisition systems.
- Fluorescent staining of the invaded cells is possible by on-plate staining of the tumoroids.

3-1 Immunostaining of tumoroids and invaded cells in the 3-in-1 Plate.

This protocol is for general immunostaining of the tumoroids.

3-1-1- Prepare 4% formaldehyde by diluting the stock 37% formaldehyde in PBS (for 10 mL, add 1.08 mL stock formaldehyde solution to 8.92 mL PBS). Aspirate the media from the Media Reservoir of the insert and add 200–300 µL of 4% solution to the reservoir and incubate at room temperature for 30 min[8].

3-1-2- Remove the formaldehyde solution and gently wash the tumoroids three times with PBS. Washing can be done by adding 200–300 µL of PBS to the Media Reservoir and incubating for 2 min to allow the materials to diffuse out of the gel into the washing solution.

3-1-3- Block the sample using BSA or normal serum related to the primary/secondary antibodies. Follow the procedure below to make the blocking buffer:

(a) Warm up PBS to 45–50 °C.

(b) Dissolve 0.3% wt/vol Triton X-100 in the warm PBS until it is clear.

[8] Fixing reagent can be chosen by the user.

(c) Weigh enough BSA to make a 5% (w/v) solution in a falcon tube. Wait until the Triton solution cools down to room temperature. Then add the 0.3% Triton solution to make the blocking buffer.

3-1-4- Aspirate the last wash solution from the Media Reservoir of the insert and add 200-300 µL the blocking buffer. Keep in the fridge overnight.

3-1-5- Make the primary antibody solution. Like the procedure for making the blocking buffer, make a solution of 1% (w/v) BSA and 0.3% (w/v) Triton in PBS. According to the protocol (for 3D culture use a decreased dilution factor), dilute the primary antibody in the 1% (w/v) BSA and 0.3% (w/v) Triton solution.

3-1-6- Aspirate the blocking buffer from the reservoir of the insert and add the primary antibody solution. 200 µL volume is enough and no washing step is required at this stage. Incubate overnight[9].

3-1-7- Remove the primary antibody solution from the Media Reservoir of the insert and wash it three times according to step 2. Subheading 3-1-2.

3-1-8- Add secondary antibody solution. To make the solution of the secondary antibody, make 1% BSA and 0.3% Triton solution similar to step 5 and dilute the secondary antibodies in it according to the supplier's protocol (for 3D culture use a decreased dilution factor). Incubate for 4–6 h in the fridge.

3-1-9- Remove the solution of secondary antibodies and add the DAPI solution. To make DAPI solution dilute stock solution of DAPI in PBS according to supplier's protocol (for 3D culture use a decreased dilution factor). Incubate for 2–3 h at room temperature.

3-1-10- Remove the DAPI solution and wash the tumoroids three times with PBS. Then, add 300 µL of PBS to the wells and the tumoroids will be ready for immunofluorescence microscopy.

[9] Steps 7 and 8 are for non-conjugated primary antibodies. If the primary antibody is already conjugated go directly to step 9.