Standard Operating Procedures for Cell-Based Assays on a Pillar Plate

This standard operating procedure (SOP) provides step-by-step methods for 3D cell-based assays on the pillar plate. Please read the protocols carefully before performing experiments.

SOPs for immunofluorescence staining of organoids on the pillar plate

Materials:

- Petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-02-00)
- 384-well plate, clear, flat-bottom (Fisher Scientific, Cat. no. 12-566-625)
- 1x PBS (Fisher Scientific, Cat. no. AAJ61196AP)
- 4% Paraformaldehyde (Fisher Scientific, Cat. no. AAJ19943K2)
- Sodium borohydride (Sigma Aldrich, Cat. no. 452882)
- Triton X-100 (Fisher Scientific, Cat. no. MTX15681)
- Tween 20 (Sigma Aldrich, Cat. no. P1379)
- Normal donkey serum (Sigma Aldrich, Cat. no. S30-M)
- Primary antibodies for target proteins of interest
- Secondary antibodies for primary antibodies
- DAPI (Fisher Scientific, Cat. no. D1306)
- Visikol Histo-M (Visikol, HM-30) or RapiClear 1.52 (Sunjin Lab, Taiwan)
- Fisherbrand™ premium cover glasses (Fisher Scientific, Cat. no. 125485J)

Methods:

1. For rinsing, insert a 36PillarPlate with organoids in the 36PetriLid on a 90 x 15 mm petri dish containing 20 mL of 1x phosphate-buffered saline (PBS), and incubate for 20 minutes at room temperature on a low-speed orbital shaker at 20 rpm.
   Note: *If the cells are encapsulated in alginate on the pillar plate, use Tris-buffered saline (TBS) instead of PBS due to alginate degradation in PBS.*
2. For fixation, prepare a petri dish with 20 mL of 4% paraformaldehyde solution, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 4% paraformaldehyde, and incubate for 2 hours at room temperature or overnight at 4°C.
3. Prepare a petri dish with 20 mL of 0.1% (w/v) sodium borohydride in PBS, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.1% (w/v) sodium borohydride, and incubate for 15 minutes at room temperature. Repeat this step once.
   Note: *This step is important to reduce background due to free aldehyde.*
4. For permeabilization, prepare a petri dish with 20 mL of 0.5% Triton X-100 in PBS, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.5% Triton X-100, and incubate for 15 minutes at room temperature with gentle shaking. Repeat this step twice to have a total permeabilization time of 45 minutes.
5. For blocking, prepare a petri dish with 5% normal donkey serum (NDS) diluted in 1x PBS with 0.25% Triton X-100 (blocking buffer), insert the 36PillarPlate in the 36PetriLid on the petri dish containing 5% NDS in the blocking buffer, and incubate overnight at 4°C or 2 - 4 hours at room temperature.

Note: *This step is critical to prevent non-specific binding of primary antibodies. You can choose to incubate either overnight at 4°C or 2 - 4 hours at room temperature depending on your application.*

6. For primary antibody staining, dilute the primary antibody solution in the blocking buffer according to the manufacturer's recommendation.
7. Dispense 80 µL/well of the diluted primary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing primary antibodies, and incubate for 24 hours at 4°C on the low-speed shaker.
8. Separate the 36PillarPlate from the 384DeepWellPlate, insert it in the 36PetriLid on a petri dish containing 20 mL of the blocking buffer, and rinse the 36PilllarPlate for 20 minutes each at room temperature on the low-speed shaker. Repeat this step twice with fresh blocking buffer.
   Note: *This rinsing step is critical to remove unbound primary antibodies.*
9. For secondary antibody staining, dilute fluorophore-conjugated secondary antibodies 200-fold in the blocking buffer, dispense 80µL/well of the 200-fold diluted secondary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing secondary antibodies, and incubate for 2 - 4 hours at room temperature on the slow-speed shaker.
10. Prepare a 384DeepWellPlate with 80 µL/well of 0.5 µg/mL DAPI in 1x PBS, insert the 36PillarPlate in the 384DeepWellPlate containing 0.5 µg/mL DAPI, and incubate for 25 minutes at room temperature with gentle shaking.
11. For rinsing, insert the 36PillarPlate with stained organoids in the 36PetriLid on a petri dish containing 20 mL of 1x PBS and incubate for 20 minutes at room temperature on the low-speed shaker. Repeat this step once.
12. For organoid clearing, dispense 30 µL/well of Visikol Histo-M or 1x RapiClear 1.52 in a clear, flat-bottom, 384-well plate, pre-warm the 384-well plate containing the clearing solution for 20 minutes at 37°C, insert the 36PillarPlate with stained organoids in the 384-well plate, and incubate for 1 hour at room temperature.
    Note: *The tissue clearing step is necessary to avoid light scattering of large organoids or spheroids and enable deep imaging of the large cell samples.*
13. For organoid imaging, separate the 36PillarPlate with stained and cleared organoids from the 384-well plate and attach the thin glass slide on the pillar plate.
14. Mount the 36PillarPlate with the thin glass slide and obtain fluorescence images using a confocal microscope.

SOPs for RT-qPCR analysis of organoids on the pillar plate

Materials:

- Sterile, RNase-free pipet tips (Fisher Scientific, Cat. no. 21-377-45, 10-320-700, and 13-611-127)
- Microplate centrifuge for detaching organoids from the pillar plate
- 70% ethanol in water
- Forward and reverse primers for the genes of interest
- Forward and reverse primers for the house-keeping gene
- RNeasy plus mini kit (Qiagen, Cat. no. 74134)
- High-capacity cDNA reverse transcription kit (Applied Biosystems, Cat. no. 4368814)
- PowerTrack™ SYBR green master mix (Applied Biosystems, Cat. no. A46110)
- Cultrex organoid harvesting solution (R&D Systems, Cat. no. 3700-100-01)
- QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Cat. no. A28574)

Methods:

1. For Matrigel degradation and organoid detachment, insert the pillar plate with organoids in Matrigel in the 384DeepWellPlate containing 80 µL of Cultrex organoid harvesting solution and incubate for 30 minutes at 4°C.
   Note: *You can collect a few organoids manually by using pipette tips in case you don't need to collect all organoids by centrifugation.*
2. Centrifuge the sandwiched plates at 100 rcf for 10 minutes using a microplate centrifuge to detach organoids from the pillar plate.
   Note: *Make sure to collect 10 - 15 replicates of organoids from the pillar plate for RNA extraction.*
3. Extract RNA from the isolated organoids using RNeasy plus mini kit.
   Note: *The detailed protocol for the use of the kit can be found on the Qiagen website.*

RNA isolation

1. Add 4 volumes of 96 – 100% ethanol to Buffer RPE to prepare a washing buffer solution.
2. Prepare a lysis buffer by adding either 10 µL of β-mercaptoethanol (β-ME) or 20 µL of 2 M dithiothreitol (DTT) in 1 mL Buffer RLT.
   Note: *β-ME or DTT in Buffer RLT is necessary for purifying RNA from cell lines or tissues containing RNases. Buffer RLT containing β-ME or DTT can be stored at room temperature for up to 1 month.*
3. For cell lysis, collect at least 10 - 15 organoids in a 1.5 mL Eppendorf tube, add 350 µL or 600 µL of Buffer RLT depending on the cell numbers, and vortex for 30 seconds for disruption and homogenization of the cells (see **Table 1** for detail).
   Note: *The organoids should be dissolved in the lysis buffer completely in 30 seconds. If not lysed completely, use a pestle to mechanically disrupt organoids. The number of organoids to be collected for RNA extraction depends on the size of organoids.*

Table 1. Volumes of Buffer RLT necessary for sample disruption and homogenization

Sample	Amount (cell #)	Cell culture dish	Buffer RLT (µL)	Disruption and homogenization
Animal cells	< 5×10^6	< 6 cm	350	Add Buffer RLT and vortex ($\leq 1 \times 10^6$ cells)
	< 1×10^7	6 - 10 cm	600	

4. Add 1 volume of 70% ethanol to the lysate from Step 3 and mix well by pipetting.
   Note: *Do not centrifuge it. Proceed to Step 5 immediately.*
5. Transfer up to 700 µL of the lysate-ethanol sample, including any precipitate, to a RNeasy mini spin column placed in a 2 mL collection tube (provided by the vendor), close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
6. Add 700 µL Buffer RW1 to the RNeasy spin column, close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
7. Add 500 µL Buffer RPE to the RNeasy spin column, close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
8. Add 500 µL Buffer RPE to the RNeasy spin column, close the lid, and centrifuge for 2 minutes at ≥ 8000 x g.
9. Place the RNeasy spin column in a new 1.5 mL collection tube (provided by the vendor) and centrifuge at a maximum speed for 1 minute to dry the membrane.
10. Place the RNeasy spin column in a new 1.5 mL Eppendorf tube (provided by the vendor), add 30 - 50 µL RNase-free water directly to the spin column membrane, close the lid, and centrifuge for 1 minute at ≥ 8000 x g to elute the RNA.
11. If the expected RNA yield is > 30 µg, repeat Step 10 using another 30 - 50 µL of RNase-free water, or using the eluate from Step 10 (if high RNA concentration is required). Reuse the collection tube from Step 10.
12. Measure the concentration of RNA using a nanodrop and prepare 1 µg RNA per 10 µL of distilled water.

cDNA conversion

1. To synthesize cDNA from 1 µg of RNA, use the high-capacity cDNA reverse transcription kit.
   Note: *The kit consists of 4 components including RT buffer, dNTP mix, RT primer, and MultiScribe reverse transcriptase. The detail protocol for the use of the kit can be found on the Applied Biosystems website.*
2. Mix 2 µL of RT buffer (10x), 0.8 µL of dNTP mix, 2 µL of RT primer (10x), 1 µL of MultiScribe reverse transcriptase, and 4.2 µL of distilled water in a PCR tube. The total volume of this mixture will be 10 µL.
3. Add 1 µg RNA in 10 µL of distilled water from Step 12 in the RNA isolation protocol in the PCR tube. The final volume of the mixture will be 20 µL.
4. Place the PCR tube in a thermocycler with the following setting: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C for ∞.

qPCR analysis

1. Perform RT-qPCR using SYBR™ Green Master Mix containing two solutions, synthesized cDNA, and primers for the genes of interest in QuantStudio™ 5 Real-Time PCR System.
   Note: *The detail protocol for the use of the kit can be found on the Applied Biosystems website.*
2. Mix 10 µL of the SYBR Green reagent, 0.5 µL of yellow solution in the kit, 1 µL of forward primer, 1 µL of reverse primer, 6.5 µL of distilled water, and 1 µL of 5 ng cDNA in a 96-well plate used for running qPCR.
   Note: *cDNA needs to be diluted in distilled water to make a final concentration of 5 ng/ µL. In addition, forward and reverse primers need to be diluted in TE buffer (Fisher Scientific, Cat. no. 12-090-015) to make a final concentration of 1 mM.*

3. Run the 96-well plate containing the samples in QuantStudio™ 5 Real-Time PCR System with the following setting: 95°C denaturation for 30 seconds, 58 - 62°C annealing for 45 seconds (depending on primer pair), and 72°C extension for 30 seconds with 35 - 45 cycles.
   Note: *Genes such as glyceraldeyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) can be used as house-keeping genes.*
4. Calculate the fold-change of relative gene expression using the delta-delta Ct method.

SOPs for compound testing with 3D cells on the pillar plate

Note: *The 384DeepWellPlate can accommodate six 36PillarPlates for compound testing. Each 36PillarPlate can be used for testing six concentrations of a compound with six replicates per concentration.*

1. Prepare a compound-in-DMSO plate with five concentrations of 4-fold serially diluted test compounds and a DMSO-alone control in a 96-well plate.
2. Prepare a compound-in-growth medium plate by diluting the compound in DMSO 200-fold with a cell culture medium and dispensing 80 µL/well of the compound solution in a 384DeepWellPlate.
   Note: *The final DMSO concentration should be below 0.5% (v/v) to avoid any basal toxicity from DMSO. Add the compound solution from low to high concentrations in the 384DeepWellPlate to avoid compound carryover. For six replicates per concentration, we will need at least 500 µL of the compound solution in the 384DeepWellPlate. Adjust the necessary compound solution volume, depending on the number of replicates and 36PillarPlates tested for different assays.*
3. Insert the 36PillarPlate in the 384DeepWellPlate with serially diluted compound solutions and incubate the sandwiched plates for 1 – 3 days in a 5% CO_2 incubator at 37°C to measure the cytotoxicity of compounds.
   Note: *Compound exposure time is determined based on the doubling time of the cells tested and the end-point assays performed.*

SOPs for cell viability assays on the pillar plate

Materials:

- Calcein AM (ThermoFisher, Cat. no. C1430)
- CellTiter-Glo 3D reagent (Promega, Cat. no. G9683)

A. Assessment of cell viability with calcein AM

1. Prepare two 384DeepWellPlates for rinsing the 36PillarPlate by dispensing 80 µL/well of DPBS in a 384DeepWellPlate.
2. Insert the 36PillarPlate with spheroids/organoids in the 384DeepWellPlate with DPBS and incubate the sandwiched plates for 15 minutes at room temperature to rinse.
3. Rinse the 36PillarPlate again with fresh DPBS in a 384DeepWellPlate for 15 minutes.
4. Prepare a cell staining plate by dispensing 80 µL/well of 1 µM calcein AM in a 384DeepWellPlate.
5. Insert the 36PillarPlate in the 384DeepWellPlate with calcein AM, wrap the sandwiched plates with aluminum foil, and incubate them for 90 minutes for spheroid/organoid staining. ***Note:*** *Stain and rinse spheroids/organoids in the dark to avoid photobleaching. Staining may take less time depending on the diameter of the spheroids/organoids.*
6. Rinse the 36PillarPlate with stained spheroids/organoids in a 384DeepWellPlate with fresh DPBS and incubate the sandwiched plates for 20 minutes at room temperature to remove excess calcein AM on the pillars.
7. Rinse the 36PillarPlate again for 20 minutes with fresh DPBS in a 384DeepWellPlate.
8. Insert the 36PillarPlate in a traditional 384-well plate with 50 µL/well of DPBS and obtain fluorescent images of the spheroids/organoids by using an automated fluorescence microscope.

B. Assessment of cell viability with CellTiter-Glo

1. Prepare a 2-fold diluted CellTiter-Glo 3D reagent according to the manufacturer's instruction.
2. Dispense the 2-fold diluted CellTiter-Glo 3D reagent into an opaque 384-well plate at a volume of 60 µL/well.
3. Insert the 36PillarPlate with spheroids/organoids in the opaque 384-well plate with the 2-fold diluted CellTiter-Glo 3D reagent, wrap the sandwiched plates with aluminum foil, and incubate them for 90 minutes at room temperature.
4. Remove the 36PillarPlate and record luminescence at an emission wavelength of 560 nm (gain at 135) from the opaque 384-well plate using a microtiter well plate reader.