Organoid Arrays on Gri3D® 96 wellplate

1. Objective

Generation of organoid arrays on Gri3D® 96 wellplate.

2. Background

This protocol describes the culturing of organoids in Gri3D® microwell arrays. The resulting organoid arrays are homogeneous and can be used for a variety of applications, such as toxicity or antibody transport assays.

3. Materials

- Gri3D® 96 wellplate of microwell size chosen (SUN bioscience) (Fig. 1),
- Basal culture medium: Advanced DMEM/F12 supplemented with 10mM HEPES, 1X penicillin/streptomycin and 1X Glutamax,
- Basal culture medium supplemented with 10% FBS,
- Organoid expansion medium (depending on organoid model),
- Cells: single cell solution from organoids expanded in Matrigel domes,
- TrypLE Express (or other dissociation reagent, depending on protocol),
- Thiazovivin or Y-27632,
- Matrigel or other ECM desired,
- 2% BSA in PBS for coating (see section 8),
- Ice.

Total recommended volume per well = **200 µl**
Cell seeding chamber = **50 µl**
Pipetting port = **150 µl**

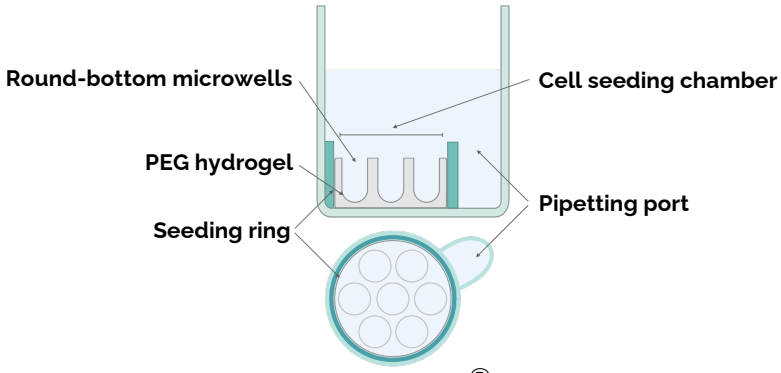

Figure 1. Gri3D® well schematic representation. Gri3D® consists of an array of U-bottom shaped microwells in a polyethylene glycol (PEG) hydrogel. The microcavities are surrounded by a seeding ring, separating the cell seeding chamber from the independent pipetting port.

4. Gri3D® plate preparation

- Collect the organoids by disrupting the Matrigel domes using ice cold basal culture medium and collect them in a 15 ml tube.
- Prepare organoid expansion medium, at least 250 µL x # wells that will be used + 20% extra (approximately 30 ml for a full 96 Gri3D® plate).
    - **IMPORTANT**: To avoid anoikis cell death, supplement the culture medium used on day 0 with a ROCK inhibitor (2.5 µM of Thiazovivin or 10 µM of Y-27632). ROCK inhibitor is not needed after the first media change (see 7).
- Before use, spray Gri3D® in its outer plastic wrapping with ethanol, open the plate under the hood, and remove the sealing layer inside the lid. Aspirate the storage buffer from the wells, both in the pipetting port and the cell seeding chamber (Fig. 2 A).
    - *TIP:* With an aspirator and a Pasteur pipette, first remove the liquid from the pipetting port. Then, carefully access the cell seeding chamber and aspirate the remaining buffer until the microwell arrays become visible (full buffer removal is not necessary). For that, slide your pipette tip on the side of the well until you feel a resistance – the seeding ring; aspirate from there without touching the hydrogel.
- Add 150 µl of organoid expansion medium in the pipetting port (Fig. 2 C). Leave the plate for 30-60 minutes at room temperature or 15-30 minutes in the incubator to equilibrate the hydrogel
    - For precious medium, carefully add 50 µl of organoid expansion medium only to the cell seeding chamber.

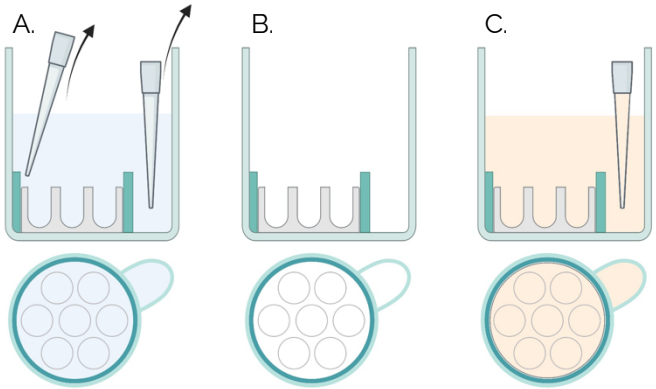

Figure 2. Gri3D® plate preparation. A-B. Remove storage buffer from the pipetting port. C. Add 150 µl of organoid expansion medium to the pipetting port and keep in incubator for 15- 30 minutes.

5. Cell suspension preparation

- Collect the organoids by disrupting the Matrigel domes using ice cold basal culture medium and collect them in a 15 ml tube.

- Centrifuge at 200 g for 4 min at 4˚C[1].
- Discard the supernatant carefully without disturbing the cell pellet and wash the organoids with 10 ml of ice-cold basal culture medium to eliminate ECM as much as possible.
- Centrifuge at 200 g for 4 min at 4˚C.
- Prepare dissociation solution. We recommend 1 ml of TrypLE Express for every 5-6 wells, and addition of 2.5 µM of Thiazovivin or 10 µM of Y-27632.
- Aspirate the supernatant and add dissociation solution. Incubate the tube at 37˚C in a water bath shaking for 5-10 minutes, make sure the organoids move constantly to guarantee an efficient dissociation. Pipette up and down the organoids with a 1000 µl every 3-5 minutes to help dissociation.
- Once a single cell solution is obtained, add 9 ml of basal culture medium supplemented with FBS to the tube to inactivate the TrypLE Express.
- Centrifuge at 200 g for 4 min at 4˚C.
- Discard the supernatant carefully without disturbing the cell pellet.
    - *OPTIONAL:* do a second wash and centrifugation with 9 mL of basal culture medium if the pellet is not clear or to wash away the FBS.
- Resuspend the cell pellet in 50-100 µL of organoid expansion medium and count the cells.
- Prepare the appropriate cell seeding density in organoid expansion medium[2]. We recommend a range of 50-200 cells in 400-500 µm microwells for human intestinal organoids, but this will need to be adapted to each model. The recommended seeding volume is 50 µl:

  Seeding density (cells/ml) = # cells per microwell x # microwells x 20

  To seed an entire plate, prepare at least 5 ml of cell suspension (5% extra volume).

Microwell diameter (in µm)	100	200	300	400	500	600	800	1000	1500
Microwell number (per well)	1677	511	211	121	73	55	31	19	7

Table 1. Microwell numbers per well as a function of the microwell diameter (in µm).

6. Cell seeding in Gri3D® microwells

- Remove organoid medium from both the pipetting port and the cell seeding chamber (Fig. 3 A-B). See page 2, tip.

[1] Centrifugation to be adapted depending on the organoid model.
[2] Seeding density will depend on the organoid model.

- Add 50 µl of cell suspension in the cell seeding chamber, on top of each microwell array (Fig. 3 C).
- Let the cells sediment for 20-30 minutes in the incubator (37°C, 5% CO2).
- Place the left over medium on ice to cool down. Thaw on ice an aliquot with the desired volume of Matrigel (or other ECM of interest).
- Once the leftover medium is cold and the Matrigel thawed, add the appropriate amount of Matrigel[3] to the medium to have 1.5-2% as final concentration (corrected with the seeded amount, 50 µl; correction factor = 1.33). Homogenize to ensure proper ECM dilution and leave the medium at room temperature.
- Take the Gri3D® plate from the incubator and check under the microscope that the cells have sedimented to the bottom of the microwells. Then, add 150 µl of medium with diluted Matrigel carefully in pipetting port (Fig. 3 D).
- Incubate the cells at 37°C, 5% CO2.

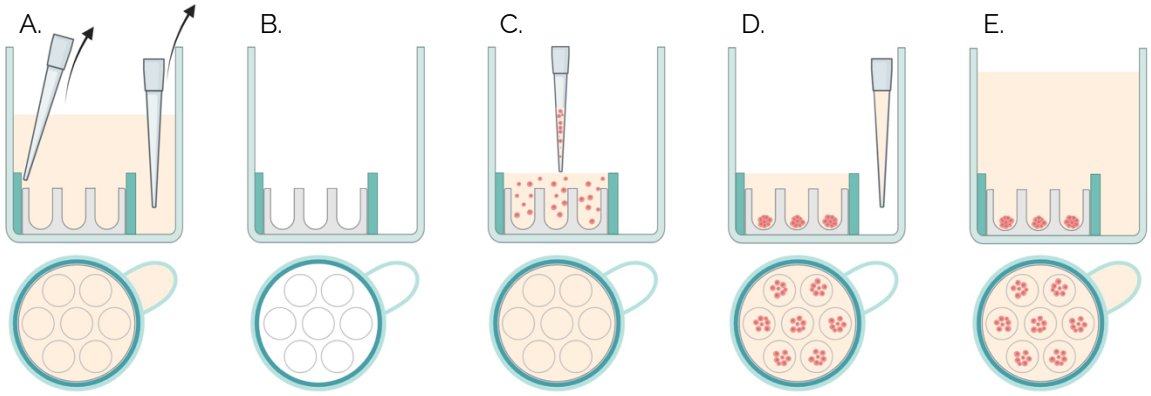

Figure 3. Cell seeding on Gri3D®. A-B. Remove medium from the pipetting port and cell seeding chamber. C. Add 50 µl of cell suspension to the cell seeding chamber. D-E. After cell sedimentation, add 150 µl of organoid expansion media to the pipetting port (with diluted ECM if desired).

7. Organoid maintenance on Gri3D®

- Change medium every 2-3 days. For that, aspirate medium from the pipetting port, and add back 150 µl of organoid expansion medium (Fig. 4). Every other medium change, add 1-1.5% Matrigel in the medium (to be adapted for each model).
- **IMPORTANT**: Do not touch the microwell array gels compartment, as that would disturb the forming organoids. Use the pipetting port instead.

[3] ECM concentration should be optimized or each organoid model.

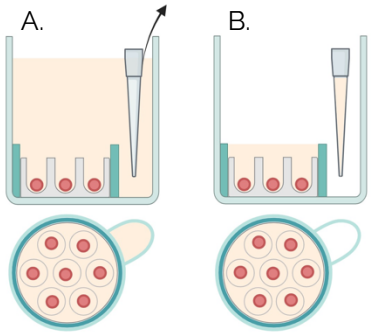

Figure 4. Cell maintenance on Gri3D®. A. Remove medium from the pipetting port. B. Add back 150 µl of organoid medium (if desired, supplemented with ECM) to the pipetting port.

8. Organoid assay on Gri3D®

- Do your assays (dye, probe, or reagent incubation) by using the pipetting port to avoid disturbing the organoid.
- If a second cell type should be added to the organoids to establish a co-culture, remove 150 µl medium from the pipetting port and carefully remove the desired volume from the cell seeding chamber (typically 20 µl). Pipette the cell solution in the cell seeding chamber on top of the organoids (typically 20 µl; cell seeding chamber maximum volume = 50 µl). Let the cells sediment for 15-30 minutes in the incubator. Add 150 µl of medium in the pipetting port and proceed with cell culture as usual (see Fig. 7).
- If organoid retrieval is required for further downstream analyses, use a 1000 µl pipette set at 150 µl approximately and pipette up and down gently in the cell seeding chamber 4-5 times (Fig. 5). The flow will allow organoids to be resuspended in the medium, which can be harvested in a tube. A washing step may be needed to make sure all organoids are recovered from the microwells.

  NOTE: organoids can stick to the pipette tips and tubes. To avoid that, we recommend pre-coating tips and tubes with 2% BSA in PBS or media. For tips, pipette the coating solution up and down a few times before collecting the organoids. For tubes, add the coating solution and leave for at least 15 minutes on ice.

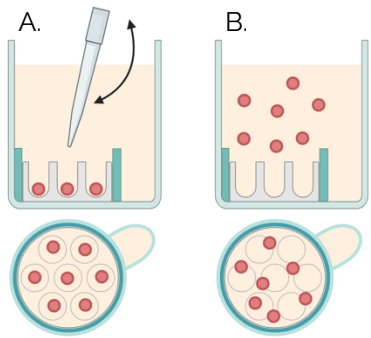

Figure 5. Organoid retrieval from Gri3D®. Use a BSA-coated 1000 µl pipette tip to resuspend the organoids by pipetting up and down in the cell seeding chamber.