Spheroid Arrays on Gri3D® 96 wellplate

1. Objective

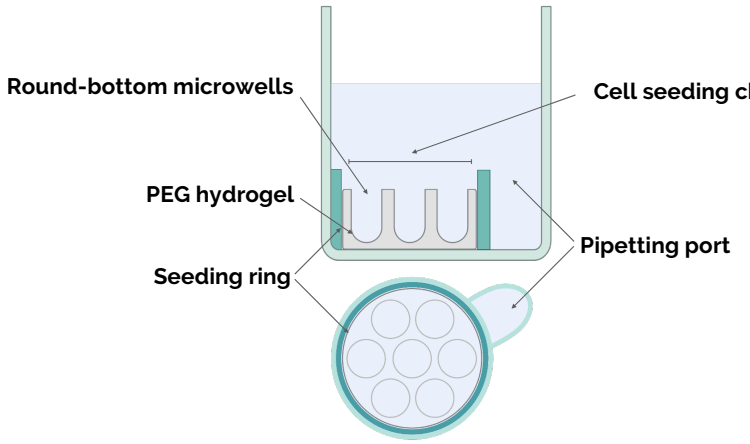
Generation of spheroid arrays on Gri3D® 96 wellplate.

2. Background

This protocol describes the culturing of spheroids in Gri3D® microwell arrays. The resulting spheroid 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),
- Spheroid medium (depending on model),
- Cells: single cell solution, starting cell numbers will depend on the model and the specificities of the experimental design,
- ECM desired (e.g., Matrigel, Cultrex, Geltrex,…),
- 2% BSA in PBS for coating (see section 8),
- Ice.



RECOMMENDATION:
Total 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

- Prepare spheroid medium, at least 250 µL x # wells that will be used + 20% extra (approximately 30 ml for a full 96 Gri3D® plate).

- 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 spheroid 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 spheroid medium only to the cell seeding chamber.***

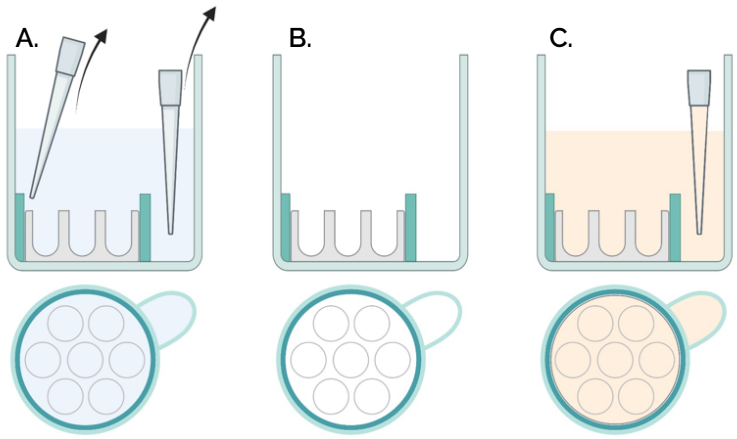


Figure 2) Gri3D® plate preparation. A-B. Remove storage buffer from the pipetting port. C. Add 150 µl of spheroid medium to the pipetting port and keep in incubator for 15- 30 minutes.

5. Cell suspension preparation

- Harvest the cells according to your in-house protocol and count.
- Prepare the appropriate cell seeding density in spheroid medium. Cell numbers may need to be optimized for each model. If aggregating different cell types, pre- mix them at the desired ratio before seeding in the microwells. 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	1600
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 spheroid medium from both the pipetting port and the cell seeding chamber (Fig. 3 A-B). See page 2, tip.
- 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).

> ***OPTIONAL:*** if ECM addition is desired, place the left over medium on ice to cool down. Thaw on ice an aliquot with the desired volume of ECM. Once the leftover medium is cold and the ECM thawed, dilute the ECM in medium. Homogenize to ensure proper ECM dilution and leave the medium at room temperature. For Matrigel, we recommend 1.5-2% as final concentration in the medium, corrected with the seeded amount, 50 µl (correction factor 1.33).

- 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 ECM carefully in pipetting port (Fig. 3 D).
- Incubate the cells at 37°C, 5% CO2.

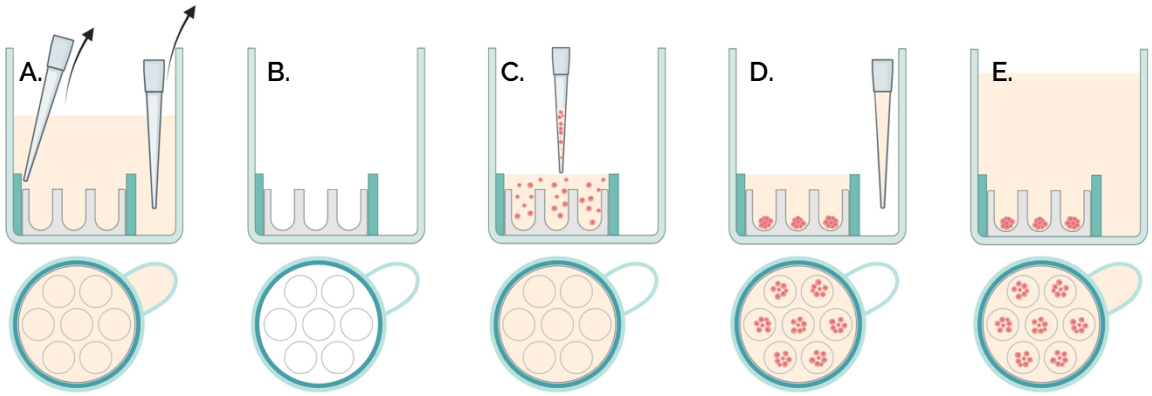


Figure 3) Cell seeding on Gri3D®. A-B. Remove medium from the pipetting port and cell seeding chamber. C. Add 50 µl of cell suspension to the cell seeding chamber. D-E. After cell sedimentation, add 150 µl of spheroid media to the pipetting port (with diluted ECM if desired).

7. Spheroid maintenance on Gri3D®

- Change medium every 2-3 days. For that, aspirate medium from the pipetting port, and add back 150 µl of spheroid medium (Fig. 4).
- **IMPORTANT**: Do not touch the microwell array gels compartment, as that would disturb the forming spheroids. Use the pipetting port instead.
    - OPTIONAL: if ECM addition is desired, add ECM at every other medium change (every 4-6 days).

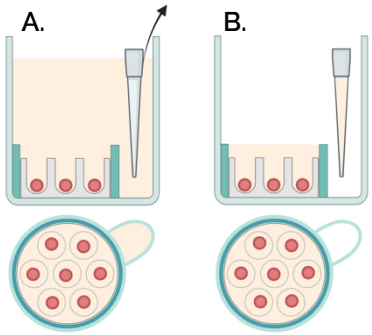


Figure 4. Cell maintenance on Gri3D®. A. Remove medium from the pipetting port. B. Add back 150 µl of spheroid medium (if desired, supplemented with ECM) to the pipetting port.

8. Spheroid assay on Gri3D®

- Do your assays (dye, probe, or reagent incubation) by using the pipetting port to avoid disturbing the spheroid.
- If a second cell type should be added to the spheroids 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 spheroids (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 spheroid 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 spheroids to be resuspended in the medium, which can be harvested in a tube. A washing step may be needed to make sure all spheroids are recovered from the microwells.

NOTE: spheroids 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 spheroids. For tubes, add the coating solution and leave for at least 15 minutes on ice.

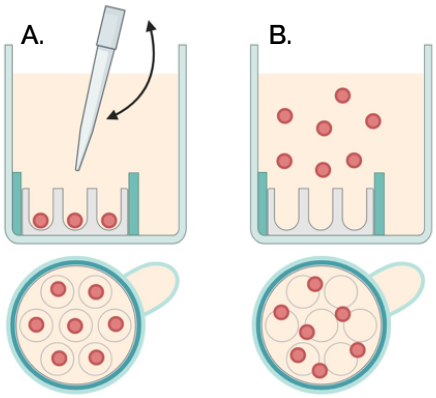


Figure 5) Spheroid retrieval from Gri3D®. Use a BSA-coated 1000 µl pipette tip to resuspend the spheroids by pipetting up and down in the cell seeding chamber.