After the digestion already carried out with SOP.

1. Wash the primary cells in a solution 3mM of EDTA + 0.05 mM DTT in PBS.

2. Prepare a solution of Matrigel (growth factor reduced BD) for each single well from 24/w plates as follows: (DMEM 10X + 5mM Hepes)/Matrigel at 1/9 ratio, specifically:

(DMEM 10X + 5m Mepes) 5.8 µl + Matrigel 52.2 µl for a total of 58µl of Matrigel solution. This dilution must be prepared by keeping all the compounds on ice.

3. 5000 primary cells are diluted in 12 µl of NSCM and mixed to 58 µl of cold Matrigel solution (this dilution is needed to maintain the concentration of DMEM 1X). The 70 µl obtained are plated in non-ultralow **24-well** plates, **previously heated**, allowing the spreading of the solution over the whole well.

4. Allow to polymerize at 37°C for at least 30 minutes.

5. Add 700 µl of NSCM + 1.4 µl of LY2109761 from the 1:10 dilution of the stock solution 1.13 mM (TGF-β receptor 1/2 inhibitor).

6. Check the wells and replace 300 ul of NSCM with the same fresh medium only when the color of the medium is metabolized.

N.B. THE ROCK INHIBITOR MUST BE ADDED ONLY IN THE NSCM AT TIME ZERO (MUST NOT BE RE-ADDED). CONVERSELY, TGF-β RECEPTOR INIBITOR AND NOGGIN MUST ALWAYS BE KEPT IN CULTURE AND ADDED EVERY 48 HOURS.

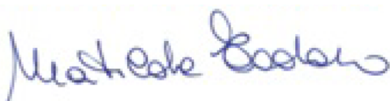
7. To calculate the number of cells needed to use a whole plate with 24 wells:

- Dilute 120,000 cells in 288 µl of NSCM.
- Prepare 1.4 ml of Matrigel solution as follows: 140 µl (DMEM 10X + 5mM Hepes) + 1.25 ml of Matrigel). This step must be carried out by keeping all the compounds on ice.
- Dilute the 288 µl of NSCM, which already contains the counted and disrupted cells as previously recommended, with 1.39 ml of Matrigel solution. Then plate 70 µl of the dilution in each individual well as already described in *point 3*.

SPHERICAL ORGANOIDS

For pharmacological or differentiation treatments

- Dissociate the spheres with trypsin or accutase.
- Wash the dissociated cells in a solution composed of 3mM EDTA + 0.05 mM DTT in PBS.



• Prepare a solution of Matrigel (growth factor reduced BD) for each single well from 24/w plates as follows: (10X DMEM + 5mM Hepes) / Matrigel at 1/9 ratio, specifically:

> (DMEM 10X + 5mM Hepes) 5.8 µl + Matrigel 52.2 µl for a total of 58 µl of Matrigel solution. This dilution must be prepared by keeping all the compounds on ice.

• 5000 primary cells are diluted in 12 µl of NSCM and mixed to 58 µl of cold Matrigel solution (this dilution is needed to maintain the concentration of DMEM 1X). The 70 µl obtained are plated in non-ultralow **24-well** plates, **previously heated**, allowing the spreading of the solution over the whole well.

• Allow to polymerize at 37°C for at least 30 minutes.

• Add 700 µl of NSCM.

• Check the wells and replace 300 µl of NSCM with the same fresh medium only when the color of the medium is metabolized.

Pharmacological or differentiation treatments can start as soon as 4- or 5-cell aggregates are visible in all the wells to be treated.

Do not forget to make the appropriate checks:

> **Control 1**: NSCM
>
> **Control 2**: NSCM + 10% FBS

To perform morphology tests, after obtaining cell growth, cut into 4 sections the matrigel of the well to be analyzed.

The individual portions must be brought together with each other as shown in the reported figure below; and thus, arranged in the enclosing box between the two sponges and fixed in buffered formalin.