Application Note

Nthy-ori 3-1 thyroid multicellular spheroids in the ClinoStar system.

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Introduction

This protocol describes the procedure for culturing spheroids of the normal thyroid cell line Nthy-ori 3-1 in the CelVivo ClinoStar system from a single cell suspension subculture.

Reagents and Materials

- Equilibrated ClinoReactor (3x)
- 1 x T175 cm^2 cell culture flask with Nthy-ori 3-1 normal thyroid cells, 70-90% confluence
- Cell counter
- Sigma Fetal bovine serum (FBS) (F7524)
- Gibco™ Roswell Park Memorial Institute (RPMI) 1640 Medium (500 ml) supplemented with 10% FBS and 1% penicillin/streptomycin
- Trypsin-EDTA (0.5%)
- 0.4% Trypan-Blue
- 70% ethanol solution
- Gibco™ DPBS, no calcium, no magnesium (14190144)
- Invitrogen™ ReadyProbes™ Cell Viability Imaging Kit, Blue/Green (R37609)
- Invitrogen™ RNA*later*™ (AM7021)

Additional Information

The general principle for cultivating spheroids is described in **01_Protocol_cultivating_Spheroids_and_Organoids** with references to relevant protocols. The cells used for the procedure were 70-90% confluent; see protocol **002_Protocol_Sub_cultivating_cells_in_2D**, which describes the sub-cultivation of cells. The protocol should be adjusted with the correct media composition as described here. The day prior to the experiment initiates the equilibration of the ClinoReactor®, see protocol **003_Protocol_Preparation_of_ClinoReactor**. The media change procedure is described in **004_Protocol_Cell_Culture_Media_Change_ClinoReactor.**

Protocol

Preparation of initial 2D cell culture

1. Nthy-ori 3-1 cells were thawed in RPMI 1640 medium (20%FBS, just for initial thawing)
2. The cell suspension was seeded into a T175 cm^2 cell culture flask
3. RPMI 1640 medium for monolayer cultures was changed every second day (48h, with RPMI 1640 media 10% FBS)
4. When the cells reached a confluence of 70-90%, they were sub-cultured
    a. The medium was removed from the cell culture flask
    b. The cell surface was washed two times with Gibco™ DPBS (14190144) without calcium and magnesium
    c. 2 mL of Trypsin-EDTA (0.5%) solution was added and the cell culture flask was placed into the incubator for 5 minutes

d. 7 mL of RPMI 1640 medium and 10% FBS was added, the cells were washed off the surface of the flask and the liquid was transferred into a 15 mL-centrifuge tube
e. The cell suspension was centrifuged at 400 × *g* for 5 minutes. The supernatant was removed and 25 mL of RPMI 1640 medium 10% FBS was used to re-suspend the cells
f. Nthy-ori 3-1 cells can be diluted up to 1:5, depending on the original confluence of the sub-cultured cell culture flask, the cells should be sub-cultured at least twice before transferring into the ClinoReactor

Culturing spheroids in the ClinoReactors

1. The cells were trypsinized for 5 min in order to be counted and then transferred to the ClinoReactors®.
2. One at least 80% confluent T175 cm^2 cell culture flask with Nthy-ori 3-1 was sub-cultured as described in the section above.
3. The cells were diluted at 1:20 in RPMI 1640 medium 10% FBS and counted.
4. 1.5×10^6 cells were seeded per previously equilibrated ClinoReactor (**003_Protocol_Preparation_of_ClinoReactor**).
5. Fresh pre-heated RPMI medium was added carefully with a syringe (10 mL) and needle 1.20 x 40 mm to maximum capacity to avoid bubbles in the cell chamber.
6. Any bubbles or clumps present were removed to avoid excessive shear forces and to maintain only spheroid culture.
7. The spheroids were monitored daily, and their rotational speed of the ClinoReactors was adjusted to counteract their increasing size. The values used are shown in Table 1 and should be understood as an example for the experiment, but not intended to be used in every culture flask. The speed should be adjusted on an individual basis.

Day of experiment	Speed (RPM)
Day 1 (seeding Day)	2.5
Day 2	3.5
Day 3	4.5
Day 4	4.5
Day 5	5.5
Day 6	6.5
Day 7 (Harvesting)	7.5

Table 1. Speed examples for growing Nthy-ori 3-1 spheroids.

8. The media was changed following the 48-72 hours scheme.
9. To change the media, the bioreactors were removed from the ClinoStar, and left in an upright position in the laminar flow bench for 5 minutes, so the spheroids can sediment to the bottom of the bioreactor.
10. Cell chambers were opened by the top plug and approximately 6 mL of medium were aspirated with syringe and needle, avoiding touching the outer membrane of the bioreactor. The removal should be slow to avoid disturbing the spheroids.
11. New pre-heated media was carefully added to the bioreactor without disturbing the spheroids. Medium was added to the very top to avoid bubbles in the cell chamber. The port was closed and 200 µl of 70 % ethanol were added to the rim to avoid contamination.

Collecting the Spheroids

1. On day 7, the bioreactors were removed from the ClinoStar and left for 5 min in the laminar flow bench in a standing position to let the spheroids sediment to the bottom of the bioreactor.
2. The bioreactor was put in the lying position and the Petri Dish was opened. 1 mL of cell suspension from each bioreactor was transferred into a 15 mL centrifuge tube.
3. 1 mL with the rest of the cells from each bioreactor was transferred into one well of a 24-well plate (other multi-well plates could also be used) for cell viability imaging.
4. The spheroids suspension in the 15 mL centrifuge tubes was centrifuged at 100 × g for 5 minutes and the supernatant was removed. Subsequently 2 mL of RNA*later*™ were added to each centrifuge tube. The tubes were later stored at 4 °C for further processing
5. Cell viability imaging - two drops of each staining (NucBlue Live for the nuclei of all cells, NucGreen Dead for the nuclei of dead cells) were added to every 1 mL well, the multi-well plate was incubated for 30 min before checking the viability under the microscope

Figures of spheroids

Below is shown how spheroids develop over time in ClinoReactors. The confocal pictures were taken every day for seven days of the experiment.

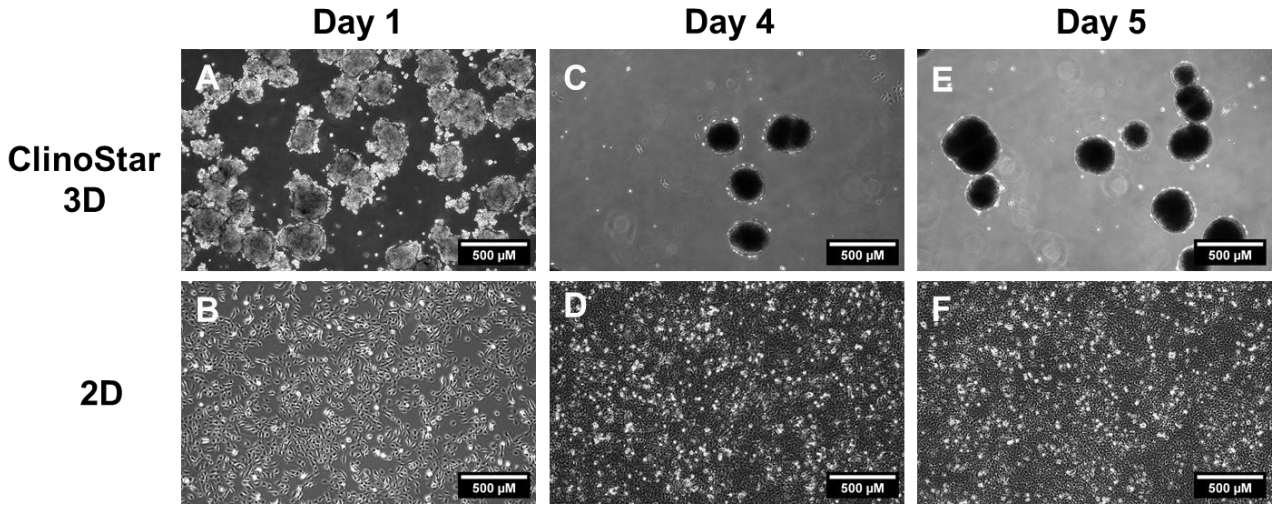

Figure 1 *Figure 1 Confocal pictures of Nthy-ori 3-1 cell line on Day 1 (A,B), Day 4 (C,D) and Day 5 (E,F) cultured in the Clinostar system and in monolayer 2D culture. Scale bar 500 µm.*

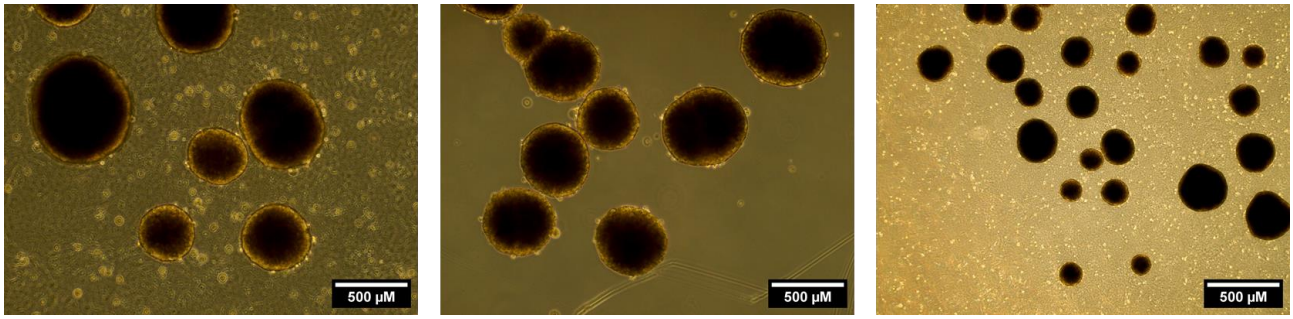

Figure 2. *Multicellular spheroids grown in the Clinostat of the Nthy-ori 3-1 cell line after 7 days. Scale bar 500 µm.*

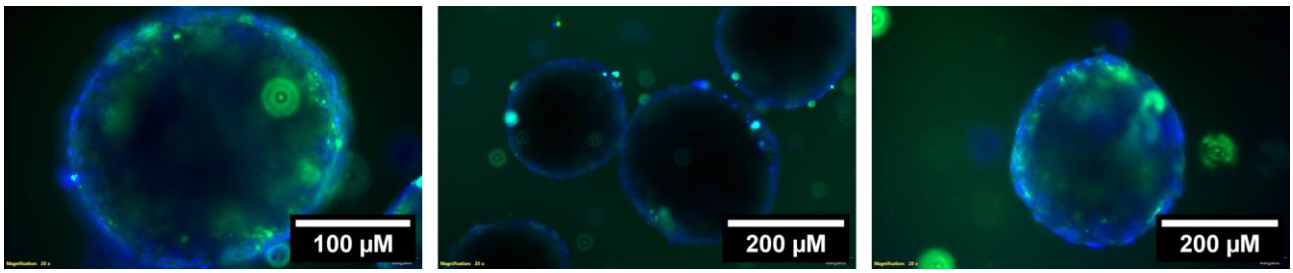

Figure 3 *Nthy-ori 3-1 cell line Live Cell Staining pictures on day 7 (NucBlue Live for the nuclei of all cells, NucGreen Dead for the nuclei of dead cells). The pictures correspond to the Live Cell Staining. We can observe that the exterior surface is composed o*

References

1. *Kopp S, Warnke E, Wehland M, et al. Mechanisms of three-dimensional growth of thyroid cells during long-term simulated microgravity. Sci Rep. 2015;5:16691.*
2. *Warnke E, Pietsch J, Kopp S, et al. Cytokine Release and Focal Adhesion Proteins in Normal Thyroid Cells Cultured on the Random Positioning Machine. Cell Physiol Biochem. 2017;43(1):257-270.*

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