SOP Number **1**
SOP Title Neurosphere cell culture

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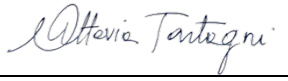
Author	**Ottavia Tartagni**	**Neurosphere cell culture**		*January 25, 2019*
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1. PURPOSE

This protocol describes how to subculture wildtype neurosphere and illustrates the specific culture conditions.

2. INTRODUCTION

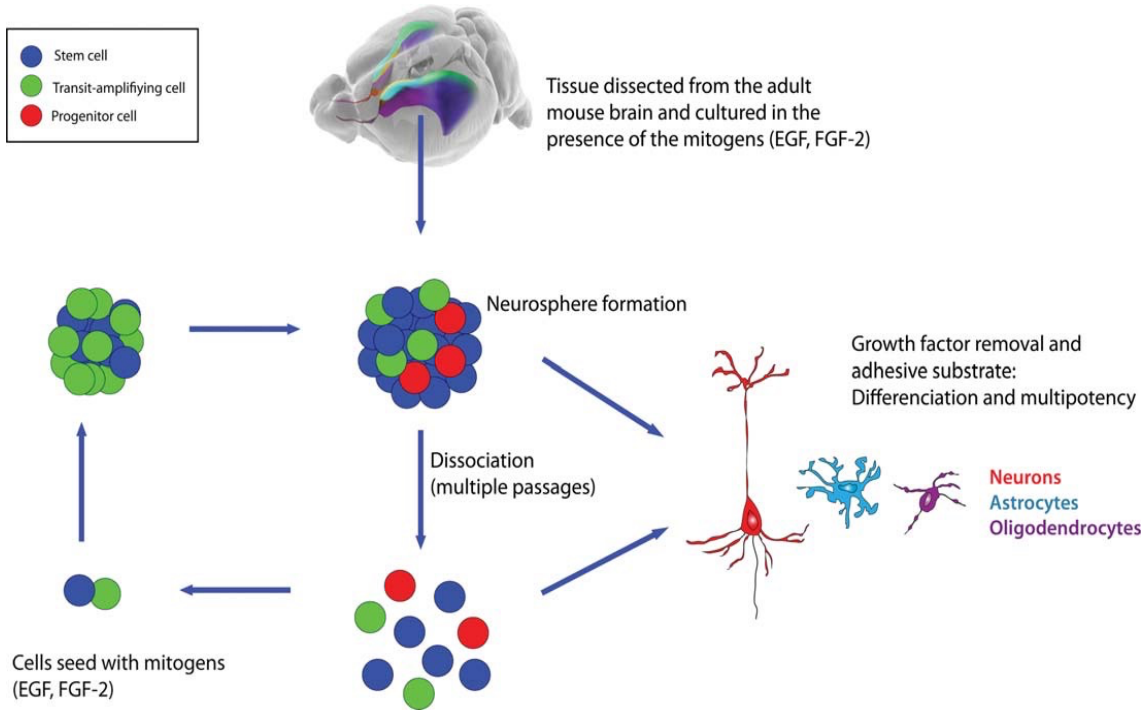
Neural stem cells from the adult SVZ have been defined in vitro by their capacity to proliferate, self-renew, and generate multipotential progeny. aNSCs (adult Neural Stem Cells) grow in vitro forming clusters that have been named as neurospheres, and therefore, the in vitro assay has been referred as “neurosphere assay.” As a model that parallels in vivo neurogenesis, the neurosphere assay has been extensively used to measure and analyze the behavior of SVZ-derived aNSCs.

The neurosphere culture is performed in a serum-free medium on untreated plates. Unknown components of serum and treatment of the plates favor irreversibly the differentiation of NSCs and prevent their self-renewal. Therefore, cell culture modifications have been developed in the absence of serum, and a defined medium has been utilized associating the addition of supplements (such as defined hormone mixes, or B7/N2 supplements). It is also crucial the availability of recombinant growth

factors, particularly epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), allowing for the maintenance of an undifferentiated state and for the induction of cell proliferation.

Hormones and other micronutrients are necessary for neurosphere culture. Insulin has neurotrophic properties and increases progenitor cell proliferation. Interestingly, progesterone, included in the defined hormone mix, has been shown to decrease proliferation in the SVZ. Transferrin is a glycoprotein that provides extracellular iron storage and transport, but also acts as an extracellular antioxidant by binding to iron and preventing it from catalyzing the production of free radicals. Glutamine is an amino acid that is shuttled between astrocytes and neurons in a process that couples ammonia metabolism to the synthesis of glutamate and aminobutyric acid (GABA) neurotransmitters. Putrescine is a low molecular weight amine that provides neuroprotection by stabilizing cellular membranes and nuclear material and is necessary for growth and replication. Selenium is an antioxidant that acts as a cofactor in glutathione production.

All these components are included in the most used commercial supplements (N2 or B27), and have to be added to basal media (DMEM F12). Regarding the dissociation method, the enzymatic method is the most commonly used. Accutase achieves higher viabilities and fast recovery after dissociation.



3. CULTURE CONDITIONS

After about 7 days from the last passage, some neurosphere will reach a considerable dimension. This conformation causes a lack of oxygen supply and leads to the formation of a necrotic core. Also, neurospheres will tend to fuse each other. This is why it is necessary to dissociate cells performing a culture passage.

In addition, every day is necessary tapping gently the dish in order to avoid neurospheres attaching to the bottom. For neurosphere growth is necessary to add EGF and FGF every other day.

4. MATERIALS

Centrifuge tube 15 ml, 50 ml
Serological Pipette 5ml, 10 ml
3.5 cm disposable petri dishes
PBS (Phosphate Buffered Saline) sterile
BSA 0.1% (Bovine Serum Albumin) (aliquot at 4°C)
Centrifuge
Incubator set at 37°C

4.1 Medium

DMEM F12 (Dulbecco's Modified Eagle Medium)
DMEM + INS + Q + PS medium
Complete Medium (DMEM+ INS + Q + PS + B27 + N2 + EGF + FGF)
Accutase (Cell Detachment Medium)

4.2 Medium Supplements

PS (Penicillin and Streptomycin) (aliquot at -20°C)
Q (Glutamine 2mM) (aliquot at 4°C)
INS (Insulin 10μg/ml) (aliquot at -20°C)
E200 (aliquot of 100μl at -80°C) > stock in powder (conc. 500 μg diluted 1:1000 in sterile H_2O)
F200 (aliquot of 10μl at -80°C) > stock in powder (conc. 500 μg diluted 1:1000 in sterile H_2O)
EGF (Epithelial Growth Factor) 1μL/ml (Stock in powder -20°C)
FGF (Fibroblast Growth Factor) 1μL/ml (Stock in powder -20°C)
N2 supplement (10μL/ml) (aliquot of 500 μl at -20°C)
B27 supplement (10μL/ml) (aliquot at -20°C)

4.3 EGF Dilution

1. Thaw an aliquot of 100μL of E200 stock (20μg/ml)
2. Add 900 μl of BSA 0.1% (aliquot at 4°C)

4.4 FGF Dilution

1. Thaw an aliquot of 10μL of F200 stock
2. Add 90μl of BSA 0.1%

5. THAWING OF NEUROSPHERES

1. Cells should be thawed rapidly by placing the cryovial in a water bath set to maintain 37°C

2. Swirl the cryovial gently in the water bath to ensure rapid thaw but do not submerge the cap of the cryovial

3. Disinfect with 70% alcohol or an equivalent disinfectant before opening

4. Using a p1000 micropipette, transfer the cryoprotectant/cells mix from the cryovial into a 15 ml falcon tube containing 1ml of DMEM F12 at 37°C. Care should be taken not to physically damage cells

5. Gently rock the 15 ml centrifuge tube back and forth while adding drops to minimize osmotic shock to the cells. This is a crucial step and cells should be treated as gently as possible.

6. Check tube to ensure all cell contents are removed

7. Centrifuge the cells at 1000 rpm for 5 min. Remove and discard the supernatant

8. Prepare a dish containing the required amount of Complete Medium

9. Take 1 ml of Complete Medium from the dish and use it to resuspend the cell pellet

10. Slowly, drop by drop, take the cell resuspension ad add it to the previously prepared dish

> *NB: Once thawed a neurosphere sample it is mandatory to monitor the culture and to add every day FGF and EGF supplements until a complete NS's size and behavior recover is observed*

6. PREPARATION OF DMEM (INS + Q + PS) MEDIUM

1. Prepare an aliquot of 25 ml of DMEM F12 medium in a falcon tube
2. Add 50μL Insulin
3. Add 500μL Glutamine
4. Add 500μL PS
5. Take off the plunger from a 50 ml syringe
6. Add a 20μm filter to the syringe
7. Verse the aliquot of DMEM F12 medium with the mentioned above supplements into the syringe barrel
8. Filter the solution into a new 50 ml falcon tube
9. Add other 25 ml of DMEM F12 medium

7. COMPLETE MEDIUM

1. Calculate the final volume of complete medium needed in a falcon tube

   NB: Usually is added 3 ml of Complete Medium for a 6 cm petri dish. Also, 1 ml of Complete Medium is required for the cell count (final volume of 4 ml for each dish of 6 cm)

2. Add FGF and EGF supplement (1μL/ml)
3. Add B27 and N2 supplement (10μL/ml)

   Warning: B27 and N2 are photosensitive supplements. Switch off the hood light before use. Moreover, all supplements must be conserved in ice out of the fridge.

8. SPECIFIC PROCEDURE

NB: Before started DMEM F12 and DMEM (INS + Q + PS) Medium should be at 37 °C, Accutase should be at room temperature

1. Begin by preparing a 6 ml aliquot of DMEM F12 medium for each dish in an empty 15 ml falcon tube.
2. Wash the dish with a 5 ml pipette, taking 1-3 ml to detach the still attached NS. Firstly, wash the corners and secondly the whole dish with left to right washings.
3. Collect in an empty 15 ml falcon tube.
4. Wash the dish with 2 ml of DMEM F12 medium- taking it from the prepared aliquot- keeping it horizontally and collect the first part, then wash with another 2 ml, collect, wash with other 2ml (final volume of 6 ml).

   Warning: Observe the dish backlit to be sure to collect as much neurosphere as possible

5. Centrifuge at 1000 rpm for 5 min at room temperature
6. Withdraw the supernatant with *p1000* micropipette and place it in the liquid containers

   Note: Do not vacuum it completely in order to not remove the pellet

7. Add 1 ml of sterile PBS using a 2 ml pipette to the falcon tube with NS pellet
8. Resuspend the pellet 4-5 times with *p1000* micropipette, aspirating and releasing firmly
9. Add 4 ml of sterile PBS without resuspending

10. Centrifuge at 1000 rpm for 5 minutes at room temperature
11. Withdraw carefully the supernatant PBS with *p1000* micropipette

Note: In order to not dilute the Accutase medium it is necessary to remove precisely the PBS solution from the neurosphere pellet

12. Add 1 ml of Accutase with a 2 ml pipette to the falcon tube with NS pellet
13. Resuspend with *p1000* micropipette 4-5 times to separate the neurosphere

Warning: This is a crucial step to appropriately dissociate neurospheres. Make sure to observe the cell resuspension, in case of visible cellular aggregates resuspend other 2-3 times

14. Leave in incubator for 5 minutes

Note: After 3 minutes move gently the falcon tube. In case of really oversized neurosphere let them in the incubator about 6-7 minutes

15. Add 4 ml of DMEM F12 medium and move without tipping the falcon

Note: The Accutase effect will be quenched by the DMEM F12 medium

16. Centrifuge at 1000 rpm for 5 minutes at room temperature
17. Prepare in a falcon tube the Complete Medium *(procedure mentioned at point 6*
18. Remove supernatant and add 1 ml of Complete Medium with *p1000* pipette
19. Resuspend forcefully 4-5 times with the p1000 set at 900 µl.

Warning: Place the tip to the wall and do not touch the bottom, push vigorously. Avoid creating bubbles.

9. NEUROSPHERE COUNT

9.1 Number of cells to plate for each type of dish

Type of dish	Area (cm^2)	Volume (ml)	Density	# cells/dish
10 cm	78,2	10	5.000c/cm^2	391.000 c/dish
6 cm	28,6	3	5.000c/cm^2	143.000 c/dish
MW 6 = 3,5 cm	9,6	2	5.000c/cm^2	48.000 c/well
MW 12	3,8	1	5.000c/cm^2	19.000 c/well
MW 24	1,9	0,5	5.000c/cm^2	9.500 c/well

Note: Generally, the number of cells to be seeded is rounded off. Example: 143.000 cell/ml > 150.000 cell/ml in a 6 cm petri dish

9.2 Procedure

1. Prepare a sterile 1,5 ml eppendorf
2. Add 10 µl of neurosphere suspension with a *p20* micropipette under the hood

   Note: First resuspend other 1-2 times with p1000 in order to count a representative cell number

3. Add 10 µl of Trypan Blue
4. Resuspend with *p20* micropipette
5. Prepare the cell counting chamber and place a cover glass
6. Inject 20 µl of the suspension in the chamber
7. Count only white cells inside each of the four chambers and calculate the average
8. Multiply the average for the dilution factor (10 µl of Trypan Blue + 10 µl of cells) and for 10.000 to calculate the number of cells for ml
9. Calculate the number of cells to subculture regarding the type of dish or multiwell to be used (see table above)

Example:

Viable cell count factor x 10.000 *(live cells per ml)* = Number Live Cells Counted / Number of large corner squares counted viable cell count X Dilution (*in this case 2)*

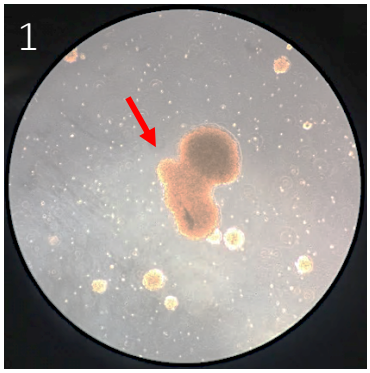
Viable cell count : 1 = 150.000 : X *(for a 6 cm dish in this case)*

X = Number of cells to subculture

Note: In order to subculture neurospheres in a specific day, it is possible to seed more or less cells to have them ready for a passage at a specific time. Example: if a neurosphere culture in a 6 cm dish was passed at day 7 but should be ready for a new passage after 6 days instead of 7, during the subculture will be seeded a range between 170.000 - 190.000 cells than 150.000.

10. MILESTONES

Figure 1:
In case of numerous oversized neurosphere around the 5thor 6th day do not add EGF and FGF supplements



Once observed a considerable amount of neurosphere oversized and fused with each other, presenting a necrotic core it will be necessary to pass the culture

Figure 2:
If the dissociation has been performed properly, during the cell count will be observed both single cells and cells aggregates

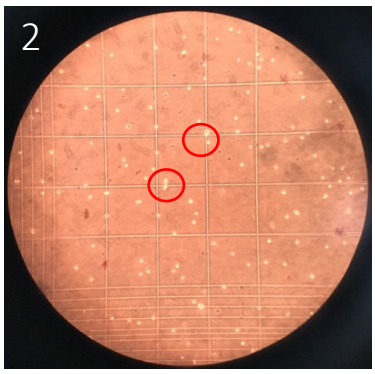


Figure 3:
If separation has not been performed properly, during the cell count will be observed intact neurospheres with only few dissociates cells. In this case it is mandatory to plate in a new dish the whole amount of neurosphere pellet with fresh Complete Medium and monitoring the culture until ready for a new passage. It is not recommended to dissociate e once again the neurosphere, this will cause cell damage.

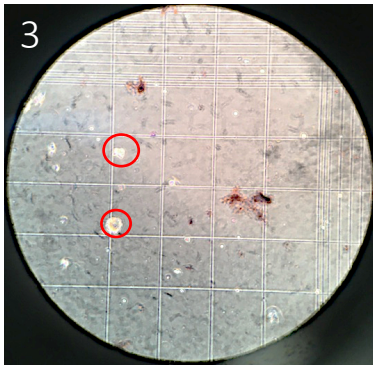


Figure 4:
Tapping gently the dish will prevent cells from getting attached to the bottom of the dish. Once neurosphere will attach to the dish they will start to differentiate losing their differentiation potential