Chapter 16

Isolation and Culture of Neurospheres from the Adult Newt Brain

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Abstract

Neural stem cells (NSCs) give rise to neurons in the adult brain and are possible targets in regenerative therapies. In vitro cultures of NSCs as neurospheres have been established from cells isolated from diverse species. Newts are exceptional regenerators among vertebrates. These animals are able to efficiently replace neurons following ablation of those by activation and subsequent differentiation of NSCs. Here we describe the method for isolating and culturing of NSCs from the newt brain both during self-renewing and differentiating conditions. Newt NSC culture provides a useful tool for functional studies of NSC fate with the potential of resulting in novel regenerative strategies.

Key words Primary culture, Neurosphere, Neural stem cells, Newt

1 Introduction

Newts have remarkable regeneration capacities among adult vertebrates [1], which extend also to the brain. In a series of experiments, it has been shown that the newt brain is able to activate sufficient cues to direct activated NSCs toward specific neuronal subtypes within an existing brain tissue [2–4]. As a complement to in vivo studies, in vitro cultures of NSCs offer additional opportunities to reveal regulatory mechanisms that control NSCs fate. A hallmark of NSCs is their potential to form neurospheres in culture. In cultures, NSC fate is easy to manipulate by over- and under-expression approaches and by treatment with small molecules. Such manipulations can be used to uncover critical regulatory mechanisms underlying proliferation and differentiation toward neurons. Recently, we used this approach to characterize the heterogeneity of newt NSCs isolated from defined brain regions at the molecular level and carry out functional manipulations of neurogenesis [5].

Here, we describe a detailed protocol for the formation and propagation of neurospheres from the adult newt brain (Fig. 1).

Anoop Kumar and András Simon (eds.), *Salamanders in Regeneration Research: Methods and Protocols*, Methods in Molecular Biology, vol. 1290, DOI 10.1007/978-1-4939-2495-0_16,

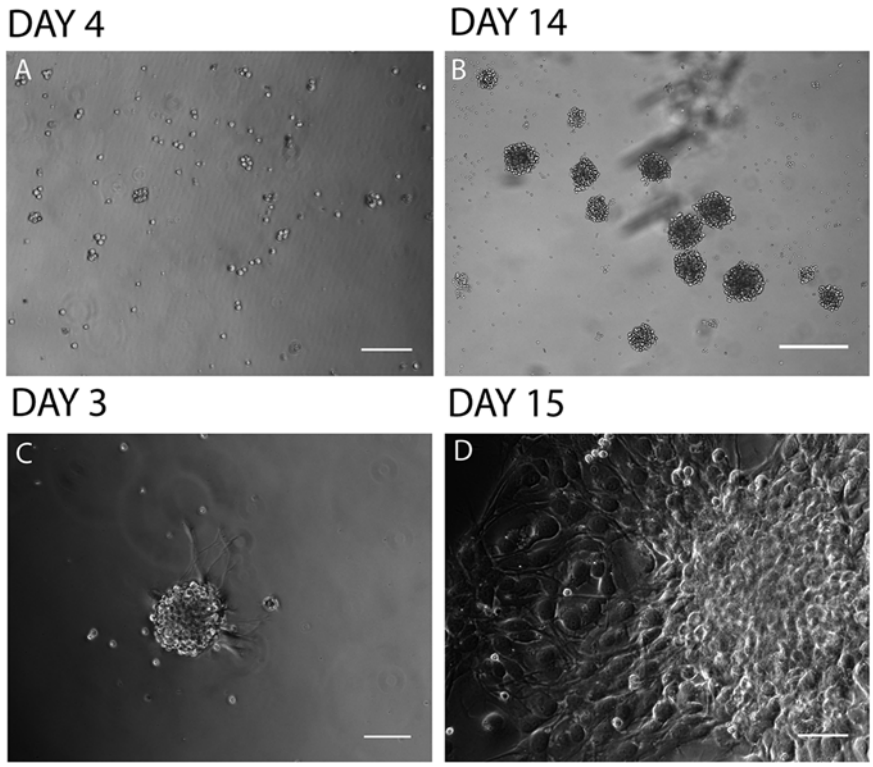

Fig. 1 Formation and differentiation of neurospheres. (**a**) Small spheres appearing 4 days after plating. (**b**) Spheres typically reach maximum size around day 14. (**c**) Plating neurospheres on PDL plates leads to attachment of the neurospheres to the substrate. (**d**) By day 15, differentiating neurospheres are apparent by bright-field microscopy

The basis of the procedure is the enzymatic digestion of newt brain into single cells. Among these single cells NSCs proliferate and form neurospheres as it has been described in other species [5]. We also outline how to promote adhesion of neurospheres to Poly-D-lysine-coated plates in order to induce differentiation of NSCs in defined culture medium (Fig. 2).

2 Materials

Prepare all solutions fresh before use. Dilute all culture medium to 66 % with Milli-Q water to adjust amphibian osmolarity (*see* **Note 1**), and filter using 0.22 μm syringe filter.

2.1 Dissection Tools and Culture Plates

1. Scalpel blade, forceps, and surgical scissors: Autoclave all instruments and sterilize with 70 % ethanol before usage (*see* **Note 2**).

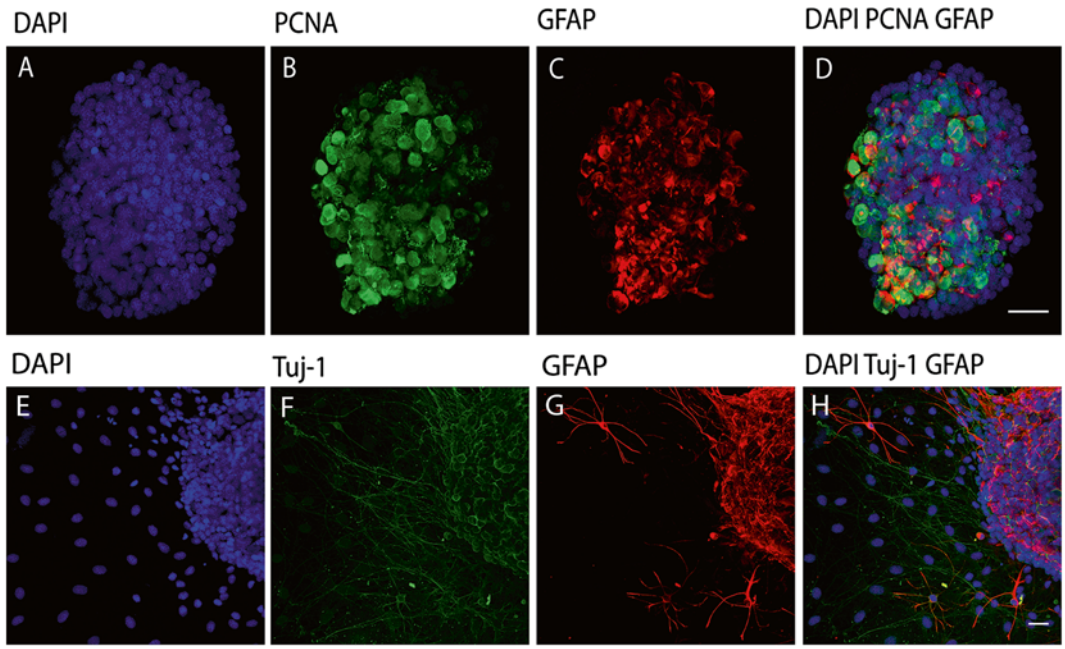

Fig. 2 Immunocytochemical characterization of neurospheres. (**a**–**d**) Growing neurospheres stained with stem cell marker GFAP and proliferation marker, PCNA. Most of the GFAP-expressing cells are marked by the proliferation marker, PCNA. (**e**–**h**) Differentiating neurospheres stained with the neuronal marker, Tuj-1, and the glial marker, GFAP. This image illustrates that most of the progeny are neurons and some progeny are glial cells

2. 8-well chamber slides, 24-well low-adherent plates, and 25 cm^2 cell culture flask.
3. 60 × 15 mm Petri dish, tissue strainer (40 μm).
4. Dissection microscope, light microscope, and fluorescence microscope.

2.2 Reagents for Neurosphere Culture

1. Anesthetic solution: Make 0.1 % of ethyl *p*-aminobenzoate (MS222) in tap water (*see* **Note 3**).
2. Dissection solution: L15 medium containing 1 % of 100 U/mL streptomycin-penicillin solution (*see* **Note 4**).
3. Enzyme solution A: Mix 30 U/mL papain and 40 μg/mL DNase in 1 mL of L15 medium.
4. Enzyme solution B: Dissolve 2 mg/mL of ovomucoid in 40 μg/mL DNase in containing L15 medium. Make 1 mL solution.
5. Enzyme digestion solution: Mix 250 μL of solution A and B, wait for 5 min (*see* **Note 5**).
6. Enzyme inhibitor solution: 2 mg/mL ovomucoid and 40 μg/mL DNase in L15.

7. Preparation of growth factors:
   (a) Dissolve 20 μg human FGF-2 in 1 mL of 5 mM Tris–HCl (pH 7.6) and sterilize through a 0.22 μm pore-size filter. Make 50 μL aliquots and store at −20 °C.
   (b) Dissolve 0.1 mg human EGF in 1 mL PBS containing 0.1 % bovine serum albumin. Make 50 μL aliquots and store at −20 °C.
8. Expansion medium: Dilute DMEM/F12/Glutamax medium to 66 % with sterile Milli-Q water and supplement with 2 % of B27 (serum-free supplement), 1 % of 100 U/mL penicillin-streptomycin, 20 ng/mL of EGF, and 20 ng/mL of FGF-2.
9. Differentiation medium: Neurobasal medium supplemented with 2 % of B27, 1 % of 100 U/mL penicillin-streptomycin, and 1 % of 2 mM Glutamax.
10. Poly-D-lysine coating (PDL): Prepare 1 mg/mL stock solution of PDL in Milli-Q water. The stock solution can be stored at −20 °C for up to 6 months. The working concentration of PDL is prepared by diluting 1 mL of stock solution in 50 mL of sterile PBS [6]. Coat the 8-well chamber slide by adding 250 μL of PDL on each well and incubate overnight at 25 °C. Wash the slides with Milli-Q water, three times for 5 min (*see* **Note 6**).

2.3 Reagents for Fixation and Staining

1. Sterile phosphate buffered saline (PBS), pH 7.4.
2. 4 % formaldehyde solution.
3. 0.5 % Tx-100 in PBS: Add 5.0 mL of Triton x-100 (Tx-100) to 995 mL of PBS.
4. Blocking solution: Add 5 mL of donkey serum to 95 mL of PBS containing 0.5 % Tx-100.
5. Primary antibodies such as anti-GFAP, PCNA, and Tuj-1. Dilute primary antibodies in blocking solution.
6. Alexa Fluor conjugated secondary antibodies. Dilute secondary antibodies in blocking solution.
7. Mounting medium: Dilute 5 mg/mL of DAPI (4′,6-diamino-2-phenylindole) by 1:1,000 in DAKO florescence mounting medium.

3 Methods

All the cell culture procedures are carried out under aseptic conditions.

3.1 Isolation of Brain Cells

1. Anesthetize newts in 0.1 % ethyl *p*-aminobenzoate (MS222). Typically it takes 20 min.

2. Sacrifice the animal and dissect out the brain under a dissection microscope.
3. Drop the extracted brain in to a 60 × 15 mm Petri dish containing dissection solution.
4. Remove carefully the meninges and blood vessels off the brain with fine forceps. Wash the tissue by transferring it to a new 60 × 15 mm Petri dish containing dissection solution (*see* **Note 7**).
5. Place the brain into fresh dissection medium and take the brain into culture hood. Use a scalpel to cut the brain into small (approximately 1 mm^3) pieces (*see* **Note 8**).
6. Collect the pieces of brain using a 1,000 μL pipette and transfer to the fresh 1.5 mL microtube. Allow the tissue to sink to the bottom by gravity, and remove the supernatant solution.
7. Add 500 μL freshly made enzyme digestion solution and incubate at room temperature for 1 h. During the incubation, stir the tube gently once every 15 min (*see* **Note 9**).
8. Add equal volume of enzyme inhibitor to stop the enzyme activity. Incubate 5 min at RT. Transfer the incubation mixture to 15 mL falcon tube and centrifuge for 5 min at 80 × *g* (*see* **Note 10**).
9. Remove supernatant and add 1 mL of fresh dissection medium to the precipitate.
10. Dissociate the newt brain cells by triturating with 1,000 μL pipette for 2 min slowly up and down (*see* **Note 11**).
11. Filter cell suspension through a 40 μm tissue strainer to remove any undissociated cells or tissue clumps.
12. Collect the filtered cells and centrifuge at 80 × *g* for 5 min.
13. Remove most of the supernatant, leaving 300 μL in the tube to make sure that cells are not lost.
14. Resuspend the cells in 4 mL of expansion medium and transfer them to a 25 cm^2 cell culture flask (*see* **Note 12**).

3.2 Neurosphere Formation and Differentiation

1. Plated isolated cells in the 25 cm^2 flask should be cultured in the incubator at 25 °C at 2 % CO_2 (*see* **Note 13**).
2. Change half of the medium once per week by centrifuging the suspension and then replacing it with half the volume of the medium (*see* **Note 14**).
3. After 14 days, collect the neurospheres by centrifuging at 80 × *g* for 5 min. These neurospheres can be either stained directly or induced to differentiate (*see* **Note 15**).
4. For differentiation, remove the supernatant, resuspend the spheres in the expansion medium, and then plate the spheres on PDL-coated slides.
5. After 24 h, when the spheres are attached, remove the expansion medium and change to neurobasal medium (*see* **Note 16**).

3.3 Immunostaining of Neurospheres and Differentiated Neurospheres

Between changing the solutions wait at least 3 min so that the neurospheres have time to resettle at the bottom of the well. Aspire the solutions by slightly tilting the plates and having the tip of the pipette touching the wall of the plate rather than the bottom [7]. Do not remove more than 75 % of the solutions.

1. Isolate the spheres by centrifugation and plate them into 24-well plates.
2. Add 4 % PFA and incubate for 15 min.
3. Remove PFA and wash three times with PBS for 5 min.
4. For proliferative cell nuclear antigen staining (PCNA), incubate neurospheres in 2 M HCl in 0.5 % Tx-100 in PBS for 20 min at 37 °C.
5. For GFAP and Tuj-1 staining, use 0.5 % Tx-100 in PBS for 15 min at RT.
6. Wash the neurospheres with PBS once for 5 min.
7. Change to blocking solution containing 5 % donkey serum in 0.5 % Tx-100 in PBS. Incubate for 30 min at RT.
8. Prepare primary antibodies in blocking buffer. Incubate with respective antibody overnight at 4 °C. Use anti-rabbit GFAP (1:500), anti-mouse PCNA (1:500), and anti-mouse Tuj-1 (1:500).
9. Wash the neurospheres with PBS once for 5 min.
10. Incubate neurospheres with Alexa Fluor conjugate secondary antibody, either Alexa Fluor 488 (1:500) or Alexa Fluor 594 (1:500) for 2 h at RT.
11. Wash the neurospheres with PBS once for 5 min.
12. Remove maximum PBS as possible, then collect the neurosphere with 1,000 μL pipette and place them on 76 × 26 mm glass slide. Add mounting medium containing DAPI on the slide and mount with a 24 × 50 mm coverslip.
13. For immunostaining differentiated neurospheres, follow all the **steps 1–12**, and omit the centrifugation steps.
14. Observe the samples under a fluorescence microscope with appropriate filters.

4 Notes

1. Amphibian osmolarity is 225 ± 5 mOsmol. Therefore, the culture medium is diluted to 66 % with Milli-Q water to match the osmolarity of the newt cells.
2. It is important to remove any remaining ethanol from the surgical tools by rinsing them with sterile PBS before touching the tissue. Traces of ethanol will act as a fixative for the tissue.

3. MS222 should be dissolved in tap water, do not use Milli-Q water.
4. Dissection solution should be kept cold during preparation of the tissue explant; therefore, keep the solution on ice. The antibiotics, penicillin, and streptomycin prevent the bacterial contamination of the cell culture medium.
5. Prepare this solution just before use.
6. PDL coating can also be done at 37 °C for 2 h. PDL-coated slides can be kept maximum for a week before using.
7. Blood vessels are a source of contamination and should be removed from the culture.
8. Smaller pieces of tissue will dissociate better and yield higher number of neurospheres.
9. Check the enzymatic digestion every 15 min and stir gently to mix the solution. The solution becomes turbid due to digestion. Make sure that there are no clumps forming during digestion. Clumping usually occurs due to the release of DNA from dead cells.
10. Digestion time varies depending on the type of tissue and concentration of enzyme. Vigorous digestion leads to fewer viable cells. Digestion is one of the most critical steps, which, if not performed correctly, would substantially reduce the number of viable cells. Also, avoid formation of air bubbles; excessive air bubbles decrease cell viability.
11. NSCs are of ventricular origin. During the digestion of the tissue, cells from the ventricular zone are released first and later other cells start to dissociate. Therefore, it is not necessary to completely digest the entire tissue.
12. The optimal cell density for neurosphere formation is about 5,000–10,000 cells/mL but may be lowered for clonal analyses.
13. Optimal culturing temperature is 25 °C; however, the cultures tolerate temperature between 20 and 27 °C.
14. Instead of changing the medium completely, it is advised to change only half of the medium at the time because prosurvival factors produced by the cells may be present.
15. Small-sized neurospheres start to appear on day 4, grow bigger over time, and reach maximum size (100–300 μm) by 2–3 weeks (Fig. 1).
16. Just removing the growth factor can be sufficient to induce differentiation of neurospheres. Defined medium leads to more robust differentiation toward various lineages. Over time, neurospheres start to differentiate and produce progenies. These cells can be stained for differentiation markers (Fig. 2).

Acknowledgment

This work was supported by a grant from the Swedish Research Council to AS.

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