Video Article

Isolation and Expansion of the Adult Mouse Neural Stem Cells Using the Neurosphere Assay

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Abstract

Isolation and expansion of the putative neural stem cells (NSCs) from the adult murine brain was first described by Reynolds and Weiss in 1992 employing a chemically defined serum-free culture system known as the neurosphere assay (NSA). In this assay, the majority of differentiated cell types die within a few days of culture but a small population of growth factor responsive precursor cells undergo active proliferation in the presence of epidermal growth factor (EGF) and/ basic fibroblastic growth factor (bFGF). These cells form colonies of undifferentiated cells called neurospheres, which in turn can be subcultured to expand the pool of neural stem cells. Moreover, the cells can be induced to differentiate, generating the three major cell types of the CNS i.e. neurons, astrocytes, and oligodendrocytes. This assay provides an invaluable tool to supply a consistent, renewable source of undifferentiated CNS precursors, which could be used for *in vitro* studies and also for therapeutic purposes.

This video demonstrates the NSA method to generate and expand NSCs from the adult mouse periventricular region, and provides technical insights to ensure one can achieve reproducible neurosphere cultures. The procedure includes harvesting the brain from the adult mouse, micro-dissection of the periventricular region, tissue preparation and culture in the NSA. The harvested tissue is first chemically digested using trypsin-EDTA and then mechanically dissociated in NSC medium to achieve a single cell suspension and finally plated in the NSA. After 7-10 days in culture, the resulting primary neurospheres are ready for subculture to reach the amount of cells required for future experiments.

Video Link

The video component of this article can be found at https://www.jove.com/video/2393/

Protocol

Part 1: Basic set up before proceeding to dissection:

1. Appropriate volume of complete NSC medium is prepared by mixing NeuroCult NSC Basal Medium and NeuroCult NSC Proliferation Supplements at a 9:1 ratio, respectively. NSC medium can also be made in laboratory based on NSC growth medium composition published in the literature[1]. If your lab does not have much experience with making medium, we strongly recommend to use commercially available medium that has been quality controled for NSCs growth prior to being sold (which is the case for NeuroCult, Stem Cell Technolgies).
2. The medium is warmed up in a 37°C water bath.
3. Cold HEPES-buffered minimum essential medium (HEM) with high concentration of antibiotics (10%) is prepared for dissection and washing purpose. Alternatively, NSC basal medium with antibiotics supplementation may also be used for this purpose.
4. 30-40 ml of cold HEM containing antibiotics is dispensed into sterile 50 ml tubes for brain collection.
5. Several 10cm plastic Petri dishes are needed to hold the brains during dissection and one 10cm sterile glass Petri dish to hold dissected tissue.
6. The surgical tools needed to remove the brain (large scissors, small pointed scissors, large forceps, small curved forceps, and a small spatula) or for tissue dissection (small forceps, curved fine forceps, and scalpel) are sterilized using glass bead sterilizer at 250°C or other available autoclave methods.
7. Dissection microscope is wiped with 70% alcohol and set up inside the PC2 hood.

Part 2: Harvesting adult mouse brain and micro-dissection:

1. 2-4 adult (5-8 weeks old) mice are anesthetized according to one's institutional approved animal protocol using 3-4% isoflurane or by intra-peritoneal injection of pentobarbital (120 mg/kg).
2. Cervical dislocation is performed to make sure the animal does not suffer pain and distress.
3. The anesthetized mouse is laid on its abdomen on an absorbent tissue paper, and the head is rinsed with 70% ethanol to sterilize the area.

4. Large scissors are used to decapitate the animal just above the cervical spinal cord region.
5. Small pointed scissors are used to make a median caudal-rostral cut and to expose the skull.
6. The cut edges of the skin are used to hold the head and then each blade of a small scissors is placed in each orbital cavity to make a coronal cut between the orbits.
7. Using the foramen magnum as an entry point, a longitudinal cut is made through the skull along the sagittal suture and two lateral cuts are also made at the junction of the lateral walls and the base of skull. Caution should be taken not to damage the underlying brain by making small cuts and ensuring the angle of the blades is as shallow as possible.
8. The skull overlying each hemisphere is grasped and peeled outward using a curved forceps to expose the brain, then using a small wetted spatula, the brain is scooped into a 50 ml tube containing HEM.
9. This procedure is repeated until all of the brains have been harvested.
10. The brains are transferred to the PC2 hood and washed three times with enough volume of cold sterile HEM to remove possible contaminants like blood and hair. The brains are then transferred to a 10cm Petri dish containing HEM.
11. To dissect the forebrain periventricular region, the dish containing the brains is placed under the dissecting microscope with low magnification. The brains are then positioned flat on their ventral surface and held from the caudal side using fine curved forceps. Another set of curved forceps is used to remove the olfactory bulbs.
12. After the removal of the olfactory bulbs, the brains are rotated to expose the ventral aspect.
13. A 90 ° coronal cut is made at the level of the optic chiasm and the caudal aspects of the brains are discarded.
14. This procedure is repeated until all brains are sectioned.
15. At higher magnification, the rostral aspects of the brains are rotated so that the cut surface faces upward. First, the septum is removed and discarded using a fine curved micro-scissors or curved, pointed forceps, and then the thin layer of tissue surrounding the lateral wall of the ventricles is cut, excluding the striatal parenchyma and the corpus callosum. Dissected tissue is pooled in a 10cm sterile glass Petri dish.
16. This procedure is repeated until all brains are micro-dissected.

Part 3: Tissue preparation and plating cells:

1. The pooled tissue is minced for about 1-2 minutes using a scalpel blade, until only very small pieces remain.
2. A total volume of 3 ml of %0.05 trypsin-EDTA is used and all of the minced tissue is transferred into a 15-ml tube. 3ml trypsin-EDTA is enough for a good digestion of tissues harvested from up to 8 mice.
3. The tube is incubated for 7 min in a 37°C water bath.
4. At the end of the enzymatic incubation, the tube is returned to the hood and an equal volume of soybean trypsin inhibitor is added to stop trypsin activity.
5. The suspension is pipetted up and down to ensure trypsin inactivation and then pelleted by centrifugation at 700 rpm (110 g) for 5min.
6. The supernatant is discarded, and the tissue pieces are resuspended in 150 µl of sterile NSC basal medium. Resetting the pipette to 200 µl, the clumps are dissociated by gently pipetting up and down (3-7 times) until a smooth milky single cell suspension is achieved. The number of pippetting steps directly depends on the size of particles in the minced tissue (it is better to mince the tissue into very small pieces so as to increase the surface area for trypsin activity). Lengthy and vigorous mechanical dissociation might lead to reduced sphere formation due to neural stem and progenitor cell death.
7. To remove debris and un-dissociated pieces, basal NSC medium is added to the dissociated cell suspension to reach a total volume of 10-15ml. The cell suspension is passed through a 40µm cell strainer into an appropriate size tube, and then pelleted by centrifugation at 700 rpm (110 g) for 5min.
8. To further remove the debris, it is recommended to resuspend the pellet in 10-15 ml of NSC medium and centrifuge the cell suspension at 700 rpm (110 g) for 5min.
9. The supernatant is discarded. Then the cells are resuspended in appropriate volume of complete NSC medium supplemented with 20ng/ml EGF, 10ng/ml bFGF and 1µl/ml of 0.2% heparin.
10. Dissociated tissue from one brain is put into one T25 flask (containing 5 ml of complete medium). The cells are then incubated at 37°C, 5% CO_2 for 7-10 days by which time neurospheres should have formed. Tissue harvested from one brain usually can generate 400-600 neurospheres.

Part 4: Passaging and expansion of NSCs:

1. When the neurospheres are ready for subculture (150-200 µm in diameter), the medium with suspended spheres is removed from the flasks, placed in an appropriate size sterile tissue culture tube, and centrifuged at 700 rpm (110 g) for 5 min at room temperature.
2. The supernatant is discarded and the spheres are resuspended in 1 ml of %0.05 trypsin-EDTA. Alternatively, neurospheres can also be passaged using mechanical or chemical dissociation methods described in the literature [2,3].
3. The cell suspension is incubated at 37°C in a water bath for 2-3 min, then an equal volume of soybean trypsin inhibitor is used to stop the trypsin activity.
4. The cell suspension is gently pipetted up and down to ensure that the trypsin has been completely inactivated.
5. The cell suspension is centrifuged at 700 rpm for 5 min. Then, the supernatant is removed and the cells are resuspended in 1 ml of NSC medium.
6. 10µl of the cell suspension is mixed with 90µl of trypan blue to perform a cell count.
7. The cells are plated at a concentration of $5x10^4$ cells/ml in complete NSC medium supplemented with 20ng/ml EGF, 10ng/ml bFGF and 1µl/ml of 0.2% heparin in an appropriate size tissue culture flask. Use 5ml medium for T25, 20 ml for T75 and 40 ml for T175 flasks.
8. Secondary neurospheres are formed in 5-7 days when incubated at 37° C in a humidified incubator with 5% CO_2.

Part 5: Representative Results:

In primary adult NSC culture, the majority of cells will die after 2-3 days but growth factor responsive precursor (including stem and progenitor cells) cells will proliferate (Figure 1) and generate colonies of undifferentiated cells after 6-8 days. In these cultures a significant amount of debris

is normally present that might acquire a spherical shape, and can be mistaken for neurospheres. The amount of debris is directly dependent on the micro-dissection and tissue preparation techniques. True neurospheres are phase bright (luminous) and become more spherical as size increases (see video). After 7-10 days, the spheres must be rounded but not compacted; and should measure between 150 and 200 µm and also should have rigid micro-spikes at the periphery at high magnification (Figure 2). If the neurospheres are allowed to grow too large, they become dark in color due to cell death at the center of the spheres. The large neurospheres are difficult to dissociate and eventually begin to attach to the substrate and differentiate.

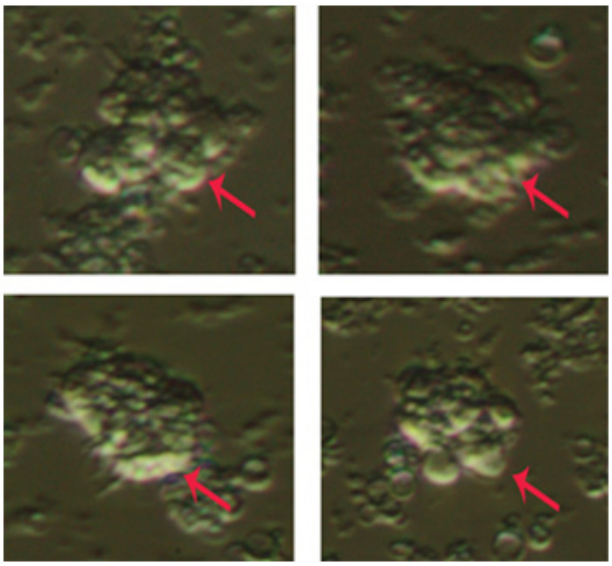

Figure 1. Primary adult NSC culture 3 days after plating. Arrows show small colonies of proliferative NSCs. Original Magnification; 20x.

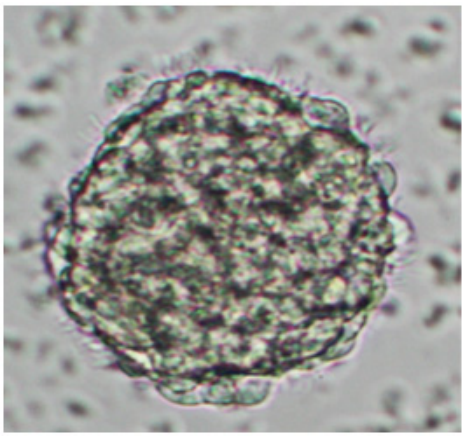

Figure 2. Passage one adult neurosphere 7 days after plating. Note the micro-spikes at the periphery of the sphere. Original Magnification; 20x.

Discussion

The neurosphere assay [2] has gained broad attention in the research community not only for the isolation and study of NSCs from CNS [4-5] but also for the isolation of other types of putative stem cells from numerous tissues, such as breast [6] and heart [7] and for the identification of brain, breast and colon tumor stem cells [8-9] suggesting that this culture system can be applied to a number of somatic and tumor precursor cell populations throughout the body. Some of the advantages of this method include its simplicity, reproducibility and generation of indefinite number of cells from a small piece of tissue or even a small number of cells in a chemically defined serum free medium.

It should be emphasized that the number of neurospheres in culture does not represent the number of stem cells as neurospheres can be derived either from bona fide stem cells or from more restricted progenitor cells [10]. In this regard an assay has been developed to correctly enumerate NSCs in culture [11].

Different methods are used to dissociate neurospheres into single cell suspension including mechanical and enzymatic methods. Although mechanical method is the original neurosphere dissociation method[2], it requires a lot of experience and its efficiency varies depending on the operator. This method also may cause significant cell death and damage if applied by an inexperienced individual, which can decrease the accuracy of the assays that rely on neurosphere dissociation[3]. Trypsin-EDTA enzymatic dissociation also has some advantages and disadvantages. Enzymatic dissociation of neurospheres is easy, efficient and reproducible if performed for the appropriate length of time and on the right sized neurospheres. Although there are no side by side studies comparing the effects of these two methods of neurosphere dissociation, our experience shows that a brief incubation (3-5 mins) of the neurospheres (up to 250 µm) with trypsin-EDTA results in an efficient

dissociation without disturbing their viability, proliferation and differentiation capabilities. On the other hand, long exposure of neurospheres (especially for the large overgrown neurospheres) to trypsin-EDTA might cause serious damage to cell surface receptors, cell digestion and potentially cell death. Overexposure to trypsin-EDTA might also interfere with sphere formation and cause cells to attach to the substrate and differentiate.

Disclosures

No conflicts of interest declared.

Acknowledgements

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References

1. Louis, S.A. & Reynolds, B.A., Neurosphere and Neural Colony-Forming Cell Assays. Protocols for Neural Cell Culture, 10, 1-28 (2010).
2. Reynolds, B.A. & Weiss, S., Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255 (5052), 1707-1710 (1992).
3. Sen, A., Kallos, M.S., & Behie, L.A., New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Eng 10 (5-6), 904-913 (2004).
4. Morshead, C.M. *et al.*, Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13 (5), 1071-1082 (1994).
5. Golmohammadi, M.G. *et al.*, Comparative analysis of the frequency and distribution of stem and progenitor cells in the adult mouse brain. Stem Cells 26 (4), 979-987 (2008).
6. Dontu, G. *et al.*, *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17 (10), 1253-1270 (2003).
7. Messina, E. *et al.*, Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95 (9), 911-921 (2004).
8. Ignatova, T.N. *et al.*, Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. Glia 39 (3), 193-206 (2002).
9. Galli, R. *et al.*, Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 64 (19), 7011-7021 (2004).
10. Reynolds, B.A. & Rietze, R.L., Neural stem cells and neurospheres--re-evaluating the relationship. Nat Methods 2 (5), 333-336 (2005).
11. Louis, S.A. *et al.*, Enumeration of Neural Stem and Progenitor Cells in the Neural Colony Forming Cell Assay. Stem Cells (2008).

