Video Article

# Isolation and Culture of Embryonic Mouse Neural Stem Cells

Farshad Homayouni Moghadam[1], Maryam Sadeghi-Zadeh[1,3], Bahareh Alizadeh-Shoorjestan[1,3], Reza Dehghani-Varnamkhasti[1,3], Sepideh Narimani[1,2], Leila Darabi[1,3], Abbas Kiani Esfahani[1], Mohammad Hossein Nasr Esfahani[1]

[1]Department of Cellular Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR

[2]Department of Biology, Faculty of Basic Sciences, Islamic Azad University

[3]Department of Biology, ACECR Institute of Higher Education

Correspondence to: Farshad Homayouni Moghadam at f_homayounim@yahoo.com

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## Abstract

Neural stem cells (NSCs) are multipotent and can give rise to the three major cell types of the central nervous system (CNS). *In vitro* culture and expansion of NSCs provide a suitable source of cells for neuroscientists to study the function of neurons and glial cells along with their interactions. There are several reported techniques for the isolation of neural stem cells from adult or embryo mammalian brains. During the microsurgical operation to isolate NSCs from different regions of the embryonic CNS, it is very important to reduce the damage to the brain cells to obtain the highest ratio of live and expandable stem cells. A possible technique for stress reduction during isolation of these cells from the mouse embryo brain is the reduction of surgical time. Here, we demonstrate a developed technique for rapid isolation of these cells from the $E^{13}$ mouse embryo ganglionic eminence. Surgical procedures include harvesting $E^{13}$ mouse embryos from the uterus, cutting the frontal fontanelle of the embryo with a bent needle tip, extracting the brain from the skull, microdissection of the isolated brain to harvest the ganglionic eminence, dissociation of the harvested tissue in NSC medium to gain a single cell suspension, and finally plating cells in suspension culture to generate neurospheres.

## Video Link

The video component of this article can be found at https://www.jove.com/video/58874/

## Introduction

Neural stem cells (NSCs) reside in different regions of the adult and embryo brain and they have a tendency to generate different types of neurons and glial cell[1]. The subventricular zones in the adult mammalian brain[2] and ganglionic eminence in the embryo brain are NSC rich regions[3]. In the developing brain, the ganglionic eminence delivers most of the cortical interneurons and particularly GABAergic interneurons[3]. There are also less invasive methods for neural stem cell generation from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) that reduce the demand for animal use. Despite the fact that generating NSCs from ESCs or iPSCs is possible[4,5], it has some advantages and disadvantages compared to the isolation of neural stem cells from the adult or embryo brain[6,7,8]. The protocols for inducing the differentiation of ESCs and iPSCs toward neuronal phenotypes are always time and cost consuming and the rate of success (70-80% Nestin positive cells)[5] is lower compared with direct isolation of NSCs from the animal brain (more than 99% Nestin positive cells)[9]. Moreover, stem cells lose their genetic stability and differentiation tendency after several passages[10,11]. Even though there are other new reports about direct conversion of somatic cells into NSCs, these cells are genetically engineered and are not easily accessible in every lab[12]. Therefore, there is still a big demand for isolation of neural stem cells from the animal brain; it is possible to reduce the quantity of animal usage by improving the surgical techniques. By reducing the surgical time and improving the techniques, it is possible to keep cells away from damage and obtain the highest rate of NSCs from each animal.

Here, we introduce a simplified and reproducible technique for isolation of neural stem cells from mouse $E^{13}$ embryo brain.

## Protocol

All the surgeries and procedures on animal were approved by the animal ethics committee of the Royan Institute, Tehran, Iran.

### 1. Preparation of Surgical Instruments, Washing Buffer (HEPES-MEM), Cell Culture Media and Cell Culture Plates

1. Sterilize the surgical tools (scissors, scalpel blade handle and forceps) by autoclaving them based on the routine sterilization guidelines.

 

2. Prepare an adequate volume of HEPES-MEM buffer (around 100 mL is enough for each pregnant mice). Add high concentrations of antibiotics (10% penicillin/streptomycin) to the HEPES-MEM buffer. This concentration of antibiotics is necessary to reduce the risk of microbial contamination.
    1. To prepare HEPES-MEM buffer, add 0.5958 g of HEPES powder to 100 mL of MEM (minimum essential medium), and mix well. Adjust the pH to 7.3-7.4 and filter the medium using a 0.22 µm filter.

3. Make NSC medium containing neurobasal medium (NB), supplemented with 20 ng/mL EGF, 10 ng/mL bFGF, 1% 100x N2 supplement or 1% B27 supplement (without vitamin A), 1 U/mL Heparin, 1% L-glutamine, 1% non-essential amino acid (NEAA), 1% Penicillin/streptomycin, and 0.1 mM β-mercaptoethanol.

## 2. Animal Surgery and Micro-Dissection

1. Anesthetize a pregnant mouse on the $13^{th}$ day of gestation (here, use BALB/c strain aged 4 months) by intraperitoneal injection of ketamine-xylazine, 100 mg/kg and 10 mg/kg by body weight respectively.
    1. Confirm deep anesthesia by exerting a painful stimulus (*e.g.,* pinch with forceps) on the animal's foot and when there is no pain reflex, sacrifice the animal by cervical dislocation.

2. Clean and disinfectant the skin of the abdomen by spraying 70% ethanol.
3. Control the skin over the abdomen and then cut the skin and the underlying fascia with scissors to uncover the abdominal cavity and uterine horns.
    1. Remove the uterine horns containing the embryos by scissors and transfer them into a 50 mL conical tube containing 25 mL of cold HEPES-MEM buffer.
    2. Place the conical tube under the laminar flow hood and open the cap under the hood. Bring the uterine horns out from the tube and rinse 3 times with enough volume of the fresh cold HEPES-MEM buffer to eliminate debris and blood.

4. Transfer the uterine tissue to a 10 cm Petri dish containing 7-10 mL of cold HEPES-MEM buffer. Open the uterine horns using fine scissors and transfer embryos to a new dish containing 7-10 mL of cold HEPES-MEM buffer. Now put the 10 cm dish with embryos under the dissecting microscope for the micro-dissection operation.
5. Bend the tip of a 25-27 G syringe needle by pushing its tip on a steel scalpel blade handle. Bend the tip of the needle until the tip is bent at an angle of 70-90°. Check the tip of the needle under the dissecting microscope to see the bend at the tip of the needle.
NOTE: Naturally, embryos have a lateral position in the dish.
6. Without changing their position, hold the neck and body of the embryo with fine forceps and then insert the bent part of the needle tip into the frontal fontanelle of the embryo head. There would be small bleeding or a blood clot there.
    1. Move the needle tip superficially. While the bent part is inside the bulge toward the forehead and then toward back of the head, make sure to cut through the skin and the skull and not to damage the underlying brain.
    2. Push the skin with the needle tip laterally to uncover the brain and insert the needle from the lateral side of the skin to the area underneath the brain. Remove the brain from the skull by pushing it to come out from the dissected skull.
    3. Hold the brain steady using the curved forceps and cut the cortex of each hemisphere using micro-scissors to expose the ganglionic eminence.
    4. Hold the brain and place the dissected ganglionic eminence into the 15 mL centrifuge tube containing neural stem cell medium. The ganglionic eminence is clearly shown with its middle and lateral parts.
    5. Repeat this procedure until all the brains have been micro-dissected.

7. Cut the dissected ganglionic eminence by placing the pipette tip against the bottom of the tube. Pipette the suspension up and down 4 to 5 times to break up the tissue for a single cell suspension.
    1. Centrifuge the suspension at 110 x g for 5 min. Remove the supernatant and re-suspend the cells in 1 mL of 0.05% trypsin-EDTA.
    2. Incubate the cell suspension in a 37 °C for 10 min, and then add an equivalent volume of soybean trypsin inhibitor (25 µg/mL) to stop the trypsin activity. Pipette the cell suspension up and down to make sure that the trypsin has been totally inactivated.
    3. Centrifuge the cell suspension at 110 x g for 5 min. Remove the supernatant and re-suspend the cells in 1 mL of complete NSC medium and count the number of cells.

8. Plate the cells ($2x10^5$ cells/mL) at NSC medium into the suitable size tissue culture flask. Transfer 5 mL to T25, 20 mL to T75 and 40 mL to T175 flasks.
NOTE: Neurospheres will appear after 3-days culture at 37 °C in a humidified incubator with 5% $CO_2$. After 6-7 days, the spheres should measure between 150-200 µm in diameter and will be ready for subculture.By cultivating one million primary NSCs after seven days the number of cells will increase to 3-4 million.

## 3. Trypsinization and Passaging of Neurospheres

1. To subculture the neurospheres, transfer the medium with suspended neurospheres to the centrifuge tube, centrifuge at 110 x g for 5 min and then discard the supernatant. Add 1 mL of 0.05% trypsin-EDTA and incubate at 37°C for 10 min.
    1. Inactivate the trypsin using soybean trypsin inhibitor and pipette the neurospheres gently up and down to make them single cells.

2. Centrifuge the cell suspension at 110 x g for 5 min, discard the supernatant and re-suspend the cells in 1 mL of NSC medium. These cells are ready for next subculture or culture on Laminin and Poly-L-Ornithine coated surfaces for advanced experiments.
3. For the next subculture, transfer NSCs into the new flask (follow step 2.8) and culture them in the NSC medium. For induction of neuronal differentiation culture NSCs in the NSC medium in the absence of bFGF and EGF on Laminin and Poly-L-Ornithine coated plates (**Figure 1B**).

4. For the coating procedure, fully coat the cell culture surface by adding enough volume of diluted poly-L-ornithine solution (25 µg/mL) to the cell culture plate and incubate at 37 °C for 2 h.
    1. Aspirate the poly-L-ornithine and rinse the well 2x with PBS. Add enough volume of 5 µg/mL laminin to completely cover the culture surface area and incubate at 37 °C for 2 h. Aspirate the laminin before plating cells or store plate at 4 °C until needed.

## 4. Flow Cytometry Assay

NOTE: The expression of specific markers of NSCs, neurons and glial cells can be measured by flow cytometry assay[13]. The protocol is based on Menon *et al.*,[13] with minor modifications.

1. Dissociate neurospheres or detach cultured NSCs from coated plates by trypsinization to make them single cells. Add 500 µL of fixation buffer (2% paraformaldehyde (PFA) in PBS) to 100 µL of the cell suspension (around 1 million cells are enough for staining with each antibody), and then incubate the tubes at RT for 15 min.
2. Wash cells by adding 1 mL of PBS to the tube and centrifuge at 110 x g for 5 min. Discard the supernatant and resuspend the cell pellet, leaving approximately 100 µL in the tube.
    1. Permeabilize cells with Tween-20 (0.7% Tween-20 in PBS) by adding 500 µL of Tween-20 buffer to 100 µL of cell suspension. Incubate the tubes at RT for 15 min on an orbital shaker (100 rpm).
3. Repeat the centrifugation and the PBS wash. Remove the supernatant from the tubes completely, leaving only the pellet behind. Add 100 µL of diluted primary antibodies (rabbit anti mouse βtub-III, GFAP and Nestin antibodies at 1:200 concentration) in dilution buffer containing 1% bovine serum albumin, 10% serum (goat serum) and 0.5% Tween-20 in PBS and gently triturate to mix. Incubate the tubes at RT for 30 min on an orbital shaker.
4. Wash the cells once with PBS and remove the supernatant from the tubes completely, leaving only the pellet behind.
    1. Add 100 µL of diluted secondary antibody (goat anti rabbit FITC conjugated at 1:500 concentration) in PBS to the cell pellet and gently triturate to mix. Incubate the tubes at RT for 30 min on a shaker in the dark.
    2. Wash the samples 2x with PBS and then wash once with flow buffer. Centrifuge and re-suspend the cells in approximately 150 µL of flow buffer for flow cytometer analysis.

## Representative Results

**Micro-Dissection, Cell Isolation and Neurosphere Culture.** Here, we presented a rapid and efficient method for mouse $E^{13}$ brain microsurgery and isolation of neural stem cells. This article shows that it is possible to remove the whole brain from an $E^{13}$ mouse embryo skull through a fine rupture in frontal fontanelle. With this method, the brain endures less damage and it is possible to isolate cells from different parts of the brain. The harvested cells could generate neurospheres in NSC medium in the presence of bFGF and EGF, and they are 150-200 µm size in diameter after seven days in culture (**Figure 1A**). Neurospheres were dissociated using 0.05% trypsin/EDTA to make them single cells and their culture on Laminin/Poly-L-Ornithine-coated dishes in the absence of bFGF and EGF showed that they extend neurite like branches and show neuronal morphology after 7-10 days (**Figure 1B**).

**Flow Cytometry Assay Results.** As shown in **Figure 2A**, 95±3.16% of the cells constituting the neurospheres were Nestin positive, which is a known marker for NSCs (**Figure 2A**). Assays on single cell cultured NSCs using β-tubulin-III and GFAP antibodies revealed that by cultivating cells on coated surfaces, the most of the NSCs will differentiate most to the neurons (94±2.67%) and less to the glial cells (5±2.46%).

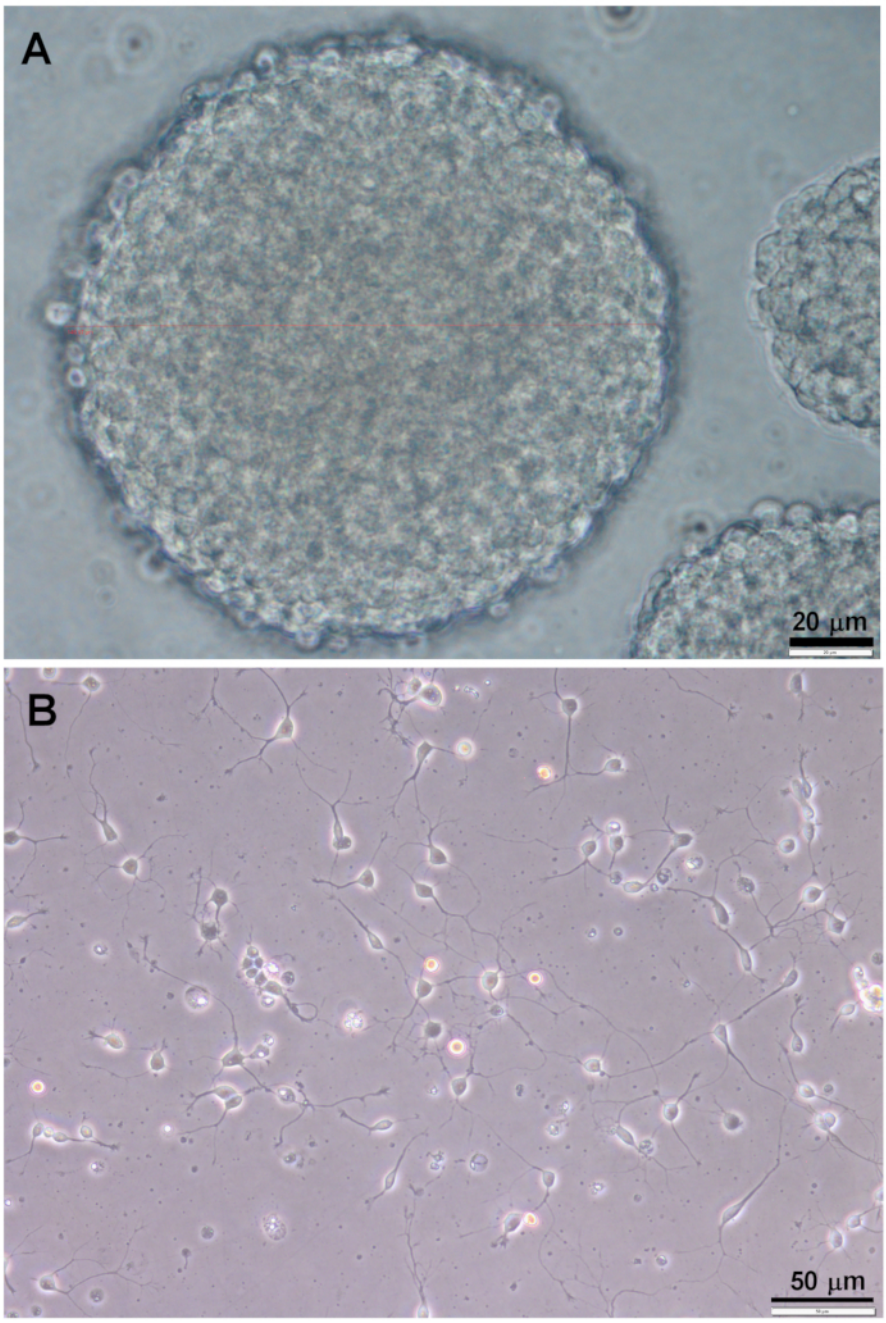


**Figure 1: Representative images from primary E[13] mouse embryo neural stem cells. A.** cultured neurospheres after 7 days. **B.** Differentiation of neural stem cells to mature neurons after 7-days culture on Laminin/Poly-L-ornithine coated dishes in NSC medium in the absence of bFGF and EGF. Scale bars represent 20 µm in A and 50 µm in B. Please click here to view a larger version of this figure.

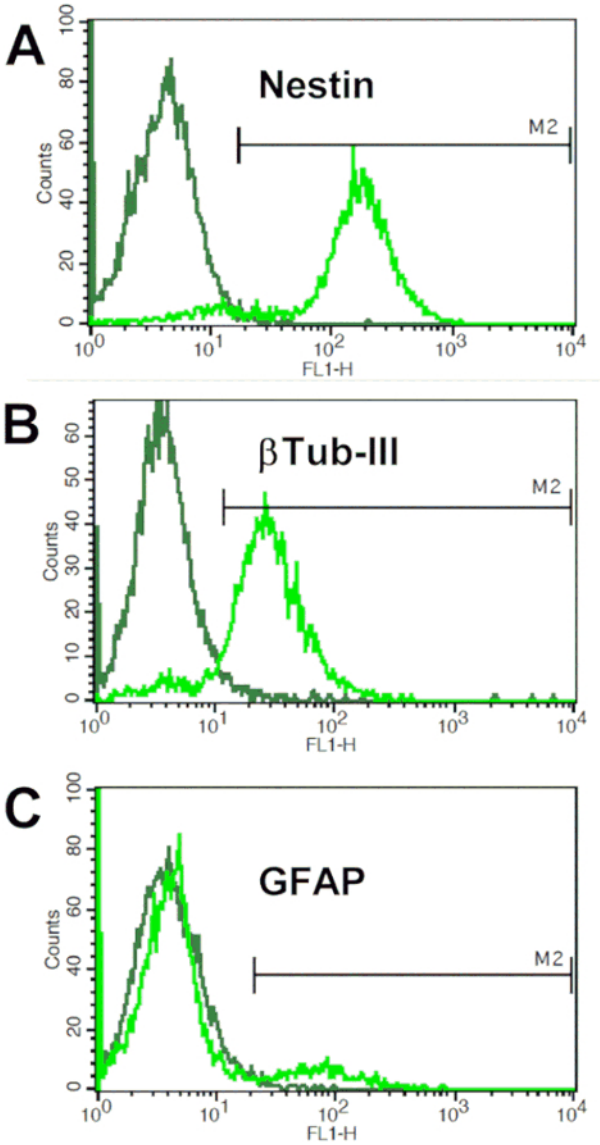


**Figure 2: The populations of Nestin (A), βtubulin-III (B), and GFAP (C) positive cells were verified by flow cytometry assay. A.** Most of the cells (95±3.16%) constructing the neurospheres were Nestin positive. **B.** Assay on adhesive cultured NSCs on Laminin/Poly-L-Ornithine coated plates in the absence of EGF and bFGF revealed that 94±3.67% of cells were βtubulin-III positive and 5±2.46% expressed GFAP. Please click here to view a larger version of this figure.

## Discussion

Using a suitable source of neural stem cells is very important for neuroscientists. Neural stem cells could be harvested from different areas of the embryo brain and they can generate specific types of neurons and glial cells. There are several methods for induction of differentiation of neural stem cells to induce them to generate mature neurons and glial cells. There are numerous reports indicating that under specific culture conditions and trophic factor regiments, they could generate neurons[14], oligodendrocytes[15,16] , and astrocytes[17] *in vitro*. Based on the data from literature, ganglionic eminence provides a good source of neural stem cells with the potential to generate different types of inhibitory and excitatory neurons such as GABAergic[18] and dopaminergic[19] types. Therefore, based on the natural tendency of these NSCs for generation of higher rate of GABAergic neurons, they provide a suitable source for studies on inhibitory neurons.

In this article, we improved the brain microdissection technique to withdraw the whole brain from the $E^{13}$ mouse embryo skull while exerting less damage to the brain tissue. To obtain the highest rate of NSCs from animal brain, it is recommendable to reduce the duration of surgical time and execute the key points such as ambient temperature and sterile conditions.

There are some critical steps for successful performance of this procedure. The animal surgery and uterine dissection must be done as quickly as possible and in a clean room. Use UV light to clean the room before surgery if available. To reduce the contamination risk, transfer uterine horns to the conical tube containing 25 mL of cold HEPES-MEM buffer and close the cap. Do not use a Petri dish in this step as it lacks a screw cap. The tube helps avoid medium leakage from the cap and helps soak the uterine horns in HEPES-MEM buffer. Put the uterine-tube under

the laminar hood and before transferring the uterine to a Petri dish, rinse uterine at least 3x with cold HEPES-MEM buffer. This step effectively reduces the risk of microbial contamination.

## Disclosures

No conflict of interest declared.

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