

In Vitro Expansion of Fetal Neural Progenitors as Adherent Cell Lines

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Abstract

In vitro studies of neural progenitors isolated from the developing mouse have provided important insights into intrinsic and extrinsic pathways that control their behavior. However, use of primary cultures or neurospheres established from fetal tissues in cell population-based assays can be compromised by cellular heterogeneity. A complementary approach that addresses this issue is the establishment of adherent clonal neural stem (NS) cell lines. Here I describe protocols and troubleshooting advice for establishing adherent NS cell lines from the mouse fetal forebrain. NS cells grow as pure cultures in defined serum-free conditions as adherent monolayers and are therefore amenable to chemical/genetic screens, biochemical studies, and population-based analysis of gene expression or transcriptional regulation (e.g. RNA-Seq and ChIP-Seq). NS cell lines therefore represent a tractable cellular model system to explore the molecular and cellular biology of neural stem cell self-renewal and differentiation. Similar protocols can be extended to rat and human embryos, as well as human brain tumors.

Key words Mouse, Development, Forebrain, Neural stem cell, Adherent, Radial glia, Epidermal growth factor (EGF), Fibroblast growth factor, Laminin

1 Introduction

Mammalian stem cell lines, such as embryonic stem (ES) cells, are an important research tool that has been used to explore the molecular basis of pluripotency and regulation of early mammalian development. Establishing permanent cell lines as a model system is advantageous as a standardized resource is generated that can be shared between laboratories. The requirement of a constant supply of embryos is also avoided through continuous propagation of stem cells in vitro. As stem cell lines can be propagated indefinitely, they are well suited to those experimental approaches which require large numbers of cells, such as genome manipulations, high throughput or high content chemical/genetic screening, biochemical analysis, and gene expression profiling.

The developing and adult mammalian nervous system contains a variety of spatially and temporally distinct neural progenitors capable of differentiation into neurons or glia. In vitro analysis of primary neural stem and progenitor cells using adherent colony forming assays [1, 2], or in suspension culture as “neurospheres” [3, 4], has provided a useful tool to explore mechanisms of self-renewal and differentiation in the developing and adult nervous system. However, to complement these studies researchers have not traditionally exploited the advantages of continuous propagation of neural stem cells as clonal cell lines, and instead typically expand primary cultures for only short periods [5].

In this chapter I describe protocols that can be used to establish and sustain clonal neural stem (NS) cell lines from mouse fetal forebrain. NS cell lines can be propagated continuously in adherent culture without the need for oncogene-based immortalization strategies [6]. NS cells have key molecular features of radial glia, long thought to provide solely an architectural role during forebrain development, but which are now known to self-renew and display neuronal and glial differentiation potential—defining properties of neural stem cells [reviewed in 7, 8].

Adherent monolayer culture has several experimental advantages that simplify analysis of stem cell behavior. Most notable is the lack of spontaneous cell death and differentiation that are a feature of neurospheres. This is analogous to ES cells, which when grown adherently are exposed to uniform culture environment and lack spontaneous differentiation, but when placed in suspension begin to generate embryoid bodies. The value of the increased homogeneity of NS cell cultures is exemplified by recent studies that have used NS cell lines to define transcription factor binding sites (e.g. REST and Mash1 [9, 10]), global mapping of epigenetic marks [11, 12], and also in studies of reprogramming to pluripotency [13–15]. Thus, NS cell lines represent a useful “discovery tool” to uncover gene function and biochemical mechanisms that control cell fate.

Despite these experimental advantages, there are important limitations that should be considered when modeling normal development and physiology. A particular concern for the use of in vitro expanded cultures is the potential loss of positional identity and consequently restrictions in the types of neurons and glia that can be produced [16]. For NS cells this is likely due to the effects of sustained exposure to FGF-2 in vitro which leads to upregulation of ventral markers such as Olig2 and Mash1 and restricts neuronal differentiation to GABAergic interneurons [17, 18]. Also, FGF-2 induces EGFR expression, which is typically found in later fetal stages, and suggests culture conditions also affect aspects temporal identity [19, 20]. To date clonal expansion of fully “plastic”

naïve neural progenitors that can be steered in vitro to any desired neuronal or glial subtype has remained elusive, although several studies have moved us towards this important goal [21–23]. Finally, as is the case with any in vitro expansion, one also needs to be cautious of the inevitable selective pressures in culture that can lead to genetic abnormalities. Thus, while there are many experimental advantages in working with in vitro NS cell lines, caution should prevail when extrapolating findings to normal development and physiology.

2 Materials

2.1 Reagents

- Dulbecco's modified Eagle's Medium/nutrient mix F-12 (DMEM/Ham's F-12) media with l-Glutamine (*see Note 1*) (PAA; E15-813) (1×).
- Glucose solution (2 M) (Sigma, G8644-100ML).
- MEM nonessential amino acids (NEAA) (PAA; M11-003 100 ml) (100×).
- HEPES buffer solution (1 M) (PAA; S11-001, 100 ml) (200×).
- Penicillin/streptomycin (PAA; P11-010, 100 ml) (100×).
- N2 Supplement (PAA; F005-004, 5 ml) (Use at half recommended; 200×).
- B27 Supplement (Invitrogen 17504-044) (Use at half recommended; 100×).
- 2-mercaptoethanol, 50 mM (Invitrogen 31350-010 20 ml).
- Bovine Albumin fraction V (BSA) solution, 7.5 % (wt/vol) (Aliquot into 10 ml stocks) (Invitrogen 15260-037, 100 ml).
- Laminin (Sigma, L2020) 1 mg/ml stock in PBS (1,000×).
- 1× Accutase solution (PAA; L11-007, 100 ml).
- Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (PAA; H15-011).
- DMSO Hybri-Max (Sigma, D26-50).
- Accutase (Sigma, A6964) (1×).
- Murine epidermal growth factor (EGF) (PeproTech EC Ltd, 315-09) 100 µg/ml stock in PBS (10,000×).
- Human fibroblast growth factor 2 (FGF-2) (PeproTech EC Ltd, 100-18B) 50 µg/ml stock in PBS (5,000×).
- BMP-4 (PeproTech, AF-120-05ET-10) 10 µg/ml stock in PBS (1,000×).
- Tissue culture plastics and associated plasticware (*see Note 2*).

2.2 Preparation

For *NS cell media* prepare 10 × 500 ml bottles of NS cell media as follows.

1. Thaw all components and bring to room temperature.
2. Use fresh stocks of sterile solutions and prepare in a tissue culture hood.
3. To each 500 ml bottle of DMEM/F-12.
 - Add 7.25 ml of sterile glucose.
 - Add 5 ml of NEAA.
 - Add 5 ml of Pen/Strep.
 - Add 2.25 ml of HEPES.
 - Add 800 µl of BSA solution (75 mg/ml).
 - Add 0.5 ml 2-mercaptoethanol (handle with care).
 - Add 2.5 ml of N2 (*see Note 3*).
 - Add 5 ml of B27.
4. Mix gently avoiding inverting bottle and store at -20°C (*see Note 4*).

“*Complete media*” (CM) is NS cell media supplemented with EGF and FGF-2 to final concentration of 10 ng/ml (*see Note 5*), as well as Laminin (1 µg/ml). We find that Laminin can be added directly to the culture media as a supplement where it is still effective at promoting attachment, avoiding the need for pre-coating of tissue culture plastic (*see Note 6*).

“*Wash media*” (WM) contains all components of the CM media, excluding N2 and B27 supplements, growth factors, and Laminin.

3 Methods

The protocols described are based on procedures using the E13.5 mouse telencephalon (*see Note 7*). However, similar cultures can be obtained from alternative developmental stages/regions or from germinal centers in the adult forebrain [6, 24] or following differentiation of mouse ES cells [6]. With some modifications they are also effective for other species (e.g., rat and human) [25, 26]. They are also applicable to certain types of human brain cancer [27].

3.1 Removal of the Mouse Fetal Brain

1. Prepare work area and dissection tools by wiping down with 70 % IMS or ethanol.
2. Using a pair of watchmakers forceps remove mouse embryos from the uterus and clean up with 2–3 washes in ice-cold PBS in a petri dish.

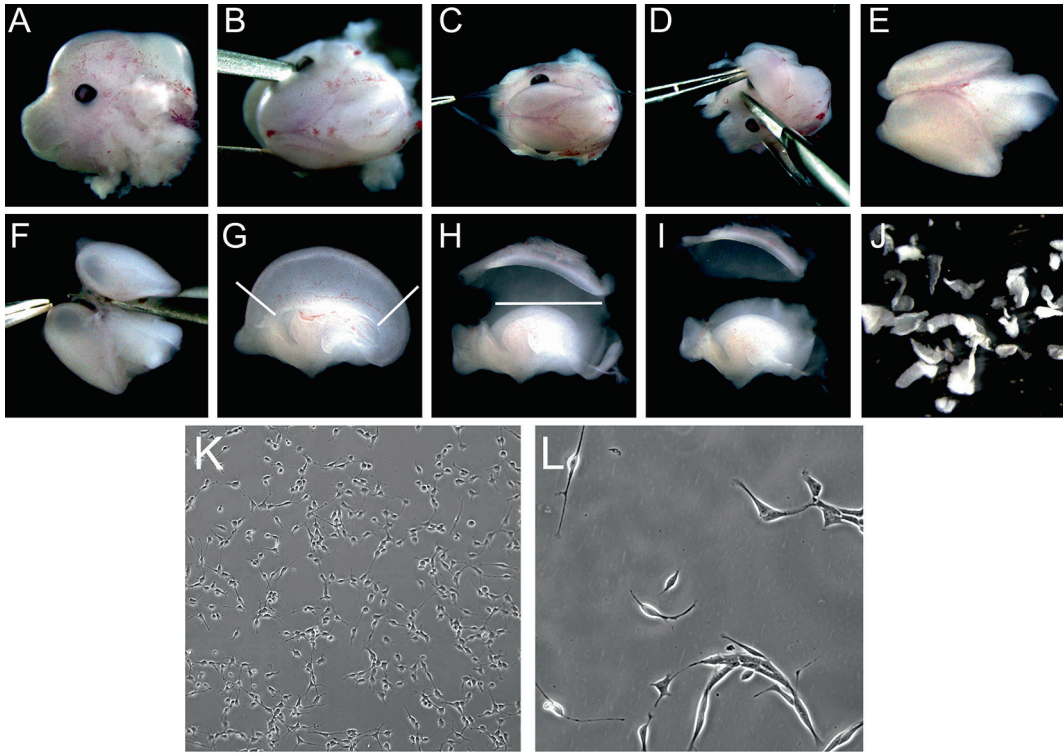


Fig. 1 Establishment of NS cell cultures from the E13.5 mouse embryo. (a)–(e) Isolation of the forebrain. (f–j) Dissection and dissociation of the dorsal forebrain. (a) Lateral view of the whole head. (b) Dorsal view of head anchored using forceps. (c) Removal of the skin. (d) Forebrain released following detachment of lateral facial tissues. (e) Isolated forebrain (telencephalic vesicles and diencephalon). (f) Separation of right telencephalic vesicle using forceps. (g) Medial surface of the telencephalic vesicle with remaining diencephalon tissues and ganglionic eminences visible. (h) Cortex released by making an incision at the rostral and caudal limits. (i) Detachment of the cortex by cutting above the dorsal edge of the LGE (*white line* in h). (j) Cortex is manually dissected into small pieces prior to dissociation in fresh PBS. (k) Appearance of NS cell cultures after several passages using phase-contrast microscopy (10×). (l) A clonal population of NS cell at higher power (20×). NS cell morphology is highly dynamic when monitored by time-lapse microscopy [6], but individual NS cells often display a typical bipolar radial glial-like morphology

3. Using forceps detach the head of each embryo and transfer to a fresh 3.5 cm petri dish filled with ice-cold PBS and place on ice (Fig. 1a).
4. Transfer one head to a fresh 10 cm petri dish filled with 10 ml of ice-cold PBS and place on dissecting microscope.
5. Anchor the head to the dish with dorsal surface facing upwards by pinning through the eye region with the forceps (Fig. 1b). Using the other forceps, pinch and then peel back the skin being careful not to damage the underlying tissues (Fig. 1c) (*see Note 8*).
6. Repeat this process to remove the dorsal portion of the developing skull.

7. Loosen the lateral developing facial tissues away from the brain by placing the forceps just below the eye and lifting/tearing caudally (Fig. 1d).
8. Separate the forebrain from the midbrain and gently lift it upwards to fully release (Fig. 1e). Discard the midbrain/hindbrain and remaining head tissues and transfer the forebrain into clean 10 cm dish containing 10 ml of ice-cold PBS.

3.2 Dissection of Dorsal Forebrain and Dissociation of Neural Progenitors

1. Release each of the telencephalic vesicles and discard the diencephalon (Fig. 1f).
2. Position each hemisphere with the medial surface facing upwards and make incisions near the rostral and caudal limits of the developing cerebral cortex (Fig. 1g).
3. Cut along the dorsal edge of the lateral ganglionic eminence to release the “flap” of cerebral cortex (Fig. 1h).
4. Remove the meningeal membranes (Fig. 1i) (*see Note 9*).
5. Transfer the isolated cortex to a fresh 3.5 cm dish (*see Note 10*), containing 1 ml of sterile PBS (Ca^{2+} and Mg^{2+} free) at room temperature and using the forceps chop the tissue into ~0.5 mm pieces (Fig. 1j).
6. Leave the tissue fragments for 5–10 min at RT to further dissociate and then triturate the undissociated tissue smoothly using a P1000 Gilson pipette for 1–2 min to achieve a single cell suspension (*see Note 11*).
7. Transfer the dissociated cell suspension to a 15 ml falcon tube containing 10 ml of pre-warmed wash media (WM) at 37 °C and mix by inversion.
8. To remove remaining non-dissociated tissue fragments pass the cell suspension through a cell strainer (75 μm) into a fresh 15 ml tube (*see Note 12*).
9. Centrifuge at room temperature for 5 min at 300 $\times g$ to obtain a pellet of dissociated cortical cells (*see Note 13*).

3.3 Establishing Adherent Neural Stem (NS) Cell Lines

1. Aspirate the supernatant and resuspend the dissociated cell pellet with 2 ml of complete media (CM) and transfer into one well of a 6-well plate. Incubate the plate at 37 °C, 5 % CO_2 (*see Note 14*).
2. Cells will expand rapidly as an adherent monolayer over the next few days (Fig. 1k). These primary cultures initially include some differentiated cells (*see Note 15*).
3. Once cells have reached ~80 % confluence (~1 $\times 10^5$ cells) they can be transferred to a T25 flask as follows.
 - (a) Remove the culture media and add 2 ml of pre-warmed PBS and remove immediately to wash away debris.

Replace this with 1 ml of pre-warmed Accutase solution (1×) and place in incubator for 2–5 min at 37 °C.

- (b) The flask or plate should can be tapped against the bench to dislodge remaining cells, and then 5 ml of WM added. Smoothly pipette the cells up and down two to three times to fully dissociate.
- (c) Transfer the dissociated cell suspension to a 15 ml falcon tube containing 5 ml of wash media and spin for 3–5 min at $300\times g$ at room temperature to obtain a pellet of dissociated NS cells.
- (d) Resuspend the cell pellet in 5 ml of CM and transfer to a fresh T25. Cells should reach confluence in 2 days and can then be continuously expanded using the same procedure (**step 3 (a)–(d)**) (*see Note 16*). Cultures should have typical NS cell appearance with individual cells displaying features of radial glia-like morphology (Fig. 1k, l).

3.4 Cryopreservation

If cells are not required immediately, then they can be cryopreserved as follows.

1. From a ~80 % confluent T25 flask ($\sim 1.5 \times 10^6$ cells) harvest a cell pellet as described above (Subheading 3.3, **step 3**). Resuspend the pellet in 1.5 ml of “freeze media” (CM plus DMSO freshly prepared to a final concentration of 10 %) and transfer 0.5 ml to each of three cryovials ($\sim 0.5 \times 10^6$ cells). Label and store at -80 °C for up to several months, or transfer to liquid nitrogen for long-term storage.
2. Cryovials can be stored at -80 °C for several months. To recover the cells a single cryovial is rapidly thawed in a water bath at 37 °C, cells are then transferred to 10 ml of pre-warmed WM, harvested by centrifugation and plated into a T25 flask containing 10 ml fresh pre-warmed complete media (CM). Following attachment (several hours or overnight) media is replaced with a fresh 5 ml CM is added to remove any residual DMSO.

3.5 Differentiation

1. For differentiation to astrocytes.
 - (a) Seed 5×10^4 NS cells into a well of a four-well plate (Nunc) in CM (without EGF or FGF-2) and supplemented with either 1 % fetal calf serum (FCS) or 10 ng/ml BMP-4.
 - (b) After 48 h >95 % of cells will have exited the cell cycle and acquire a characteristic stellate morphology.
2. For differentiation to neurons (*see Note 17*).
 - (a) Seed 5×10^4 NS cells into each well of a four-well plate (Nunc) in CM without EGF, but plus FGF-2 at 10 ng/ml (i.e. EGF withdrawal).

- (b) The following morning exchange the media fully with CM supplemented with FGF2 (5 ng/ml) + an additional 0.5× B27 supplement (Gibco).
- (c) After 4–5 days exchange medium to NS basal media mixed with Neurobasal media (Invitrogen) (1:1) and supplement with 0.5× B27 (Gibco) but no growth factors. Cells with neuronal morphology should emerge over the next 3–7 days. Replace half media with fresh every 3–4 days.
- (d) In order to maintain neurons for longer periods, from day 14 of differentiation switch media to Neurobasal media supplemented with B27 + BDNF (10 ng/ml; R and D systems), but without N2. Replace half of the media with fresh every 3–4 days. Such conditions should enable neuronal survival for a further 3 weeks [28].

3.6 Clonal Analysis

NS cell cultures can be used to establish clonal lines through plating at low density or single cell deposition in 96 well plates. Colony formation also serves as a useful assay for quantitative analysis of self-renewal.

1. Dispense 5,000 cells into a 10 cm dish in CM.
2. Around 10 days later around 200–300 discrete NS cell colonies will appear with around 1,000–2,000 cells/colony (*see Note 18*).
3. Colonies can be “picked” for further expansion as clonal cell lines as follows.
 - (a) Remove the culture media from the 10 cm dish leaving only ~0.5 ml.
 - (b) In the tissue culture hood hold up the plate up and tilt to 45° so that the residual media accumulates at the bottom. Rotate the plate such that the desired colony is free from media.
 - (c) Load a P20 pipette with 20 µl of CM, use the tip to scratch in the region of the colony (*see Note 19*). Pipette the media up and down quickly once to dislodge and capture the colony.
 - (d) Transfer these cells into one well of a 48-well plate and allow cells to expand until near confluent before passaging into a six-well plate and subsequently T25 flask.

3.7 Transfection (Nucleofection)

The nucleofection protocol described below can be used to achieve 50–70 % transient transfection of plasmid DNA using the Amaxa nucleofector™ (Lonza). It can also be adapted to generate stable transgenic clonal lines if used with linearized plasmid DNA and antibiotic selection. NS cells can also be genetically modified through use of viral vectors (retro- or lentiviral based).

1. Transfection requires $1\text{--}5 \times 10^6$ cells and 2 µg of plasmid DNA.

2. Add 0.5 ml of Amaxa supplement to 2.25 ml of Nucleofector solution V (as manufacturers instructions) and mix. This solution is now stable for 3 months at 4 °C.
3. Pre-warm Nucleofector solutions at room temperature.
4. Pre-warm NS media +10 % serum for cell recovery.
5. Prepare plates and media, label and place in incubator to warm.
6. Detach NS cells using protocol described above (Subheading 3.3, **step 3**) and centrifuge to harvest cells.
7. Resuspend the pellet in appropriate amount of nucleofection solution V (100 µl for each reaction, 1×10^6 cells). Do not leave longer than 15 min. Add 2 µg of DNA/reaction to this. Cells are now ready for nucleofection.
8. Transfer 100 µl cell solution to cuvette (*see Note 20*).
9. Choose program T-030 (T-020 will give better survival but less efficient).
10. Add cuvette to holder and press X to start.
11. Transfer cells gently into plates, transfer to the cell culture incubator and allow them to attach.
12. Once attached (usually ~2–3 h later), exchange media with fresh CM, washing twice to remove traces of serum.
13. Add drug selection after at least 24 h, or sort cells using flow cytometry (if a fluorescent reporter has been used).

4 Notes

1. For live fluorescence imaging DMEM:F12 should be replaced with Eagles modified essential media (EMEM, Sigma, M2645-10L) have less riboflavin, which significantly reduces background autofluorescence.
2. We find that tissue culture plastics supplied by Iwaki are optimal for attachment of NS cells.
3. Ensure that the supplement is fully in solution by pre-warming in water bath to 37 °C. Problems with a decrease in general viability of cells have been observed due to reduced insulin activity within the N2 supplement. N2 can also be “home made” as described previously [29].
4. *Optional*: to test for sterility, remove a 10 ml aliquot and mix with Tryptose solution 1:1 and place in incubator. This solution will turn cloudy within 1–2 days if contaminated with bacteria.
5. EGF and FGF-2 are stored in 50 µl aliquots at –20 °C (at 100 µg/ml and 50 µg/ml stock solutions in PBS, respectively) and are added freshly to the culture media. Thawed vials can be stored at 4 °C and should be used within 2–3 weeks.

6. This reduces the need for “pre-coating” of plates and reduces costs (coating is 10 $\mu\text{g}/\text{ml}$ 2–3 h incubation; when used as a supplement 1 $\mu\text{g}/\text{ml}$ is needed). For cell lines that are prone to aggregate increasing laminin in the culture media to 2 $\mu\text{g}/\text{ml}$ can often promote monolayer expansion. We have found Laminin from different suppliers can vary dramatically in effectiveness. Laminin-1 as supplied by Sigma (L2020) has proven most consistent.
7. Although an E13.5 embryo is illustrated in this protocol, similar NS cells have been established from any stage between E12.5 and E18.5 using the same dissection/protocol. If working with a specific transgenic strain, then each embryo must be kept separate during the whole procedure. The remaining tissues after removal of the brain should be kept for genotyping. We normally process one litter at a time (10–15 embryos dissected within 1–2 h). We do not routinely use a laminar flow hood for the dissections.
8. The embryo can be anchored by placing each prong of the forceps into the eye and pinning down onto plastic and this makes it easier to make the initial pierce in the central region of the developing skull; after E15.5 the skull is more developed and care should be taken to avoid damaging the underlying brain.
9. The meningeal membranes are more difficult to detach at E12.5. It can help to leave the tissue in PBS for 5 min before removal.
10. For transferring tissue pieces use a P1000 and cut the tip with scissors to increase the diameter to ~ 2 mm.
11. Do this smoothly avoiding air bubbles as much as possible as this will result in reduced cell viability. Remove around 800 μl at a time and “blast” against the bottom of the dish to promote dissociation.
12. For establishing NS cell lines full dissociation of the tissue is not required. However, for detailed studies of the full repertoire of primary progenitors, then enzyme-based dissociation protocols should be used to maximise recovery of the full cellular population [30].
13. A more defined/compact cell pellet is obtained by spinning down cells in wash media than using PBS.
14. The same media and handling of cells can be applied to differentiating ES cells to generate NS cells [6, 29].
15. If there is extensive debris or the cell density is high then cells will initially form aggregates or neurospheres. These can be harvested by centrifugation at $100 \times g$ for 30s and placed into a fresh well, where attachment should occur within 24 h

(pre-coating of plastic with the Laminin can be used; 10 µg/ml in PBS at 37 °C for 3 h).

16. We typically expand the cultures up to passage 3 or 4 before freezing down stocks. At this point >99 % of cells are immunopositive for nestin, RC2, Olig2, and Sox2 [6]. NS cells can then be continuously expanded. Mouse NS cell lines are effectively immortal, and have been continuously expanded for over 100 passages without crisis or changes in differentiation potential [6]. However, as with any in vitro culture with higher passage numbers there is a risk of accumulating genetic defects [31]. It is recommended that a large number of vials are stored at early passages and experiments past passage 20 cultures are avoided where possible.
17. Modifications to the neuronal differentiation protocol have been reported that enable more efficient generation of oligodendrocytes [32]. Differentiation works best using tissue culture plastic precoated with Laminin.
18. For true clonal analysis single cells should be deposited into individual wells using a cell sorter or limiting dilution and visual inspection of wells. Efficient colony formation depends on B27 supplement and is much less efficient using only N2. Precoating with Laminin can help restrict the colony size.
19. Areas with colonies can be highlighted with a marker pen on the bottom of the flask in advance to help picking.
20. Add solution gently and do not mix, just withdraw cells smoothly and place into a 1.5 ml microfuge tube for 5 min.

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Methods and Protocols

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