Mouse Neural Progenitor Culture (Neurosphere Assay)

In vitro culture of neural stem/progenitor cells from embryonic mouse brain

Overview

The neurosphere assay is a method for culturing neural stem and progenitor cells (NSPCs) from embryonic or postnatal mouse brain. Cells are grown in suspension as free-floating aggregates (neurospheres) in the presence of EGF and FGF growth factors.

Workflow Summary:

- **Step 1:** Dissect telencephalic vesicles from E13-E15 embryos
- **Step 2:** Chemical dissociation (10 min at 37°C)
- **Step 3:** Trituration to single cells
- **Step 4:** Wash and resuspend in complete stem cell medium
- **Step 5:** Culture in ultra-low attachment dishes
- **Duration:** Neurospheres form in 48-72 hours
- **Passaging:** Dissociate and replate every 5-7 days

Applications

- Neural stem cell self-renewal studies
- Differentiation assays (neurons, astrocytes, oligodendrocytes)
- Drug screening on neural progenitors
- Developmental neurobiology
- Brain tumor stem cell studies
- Neurogenesis research

Key Features

- Maintains stem cell characteristics
- Self-renewing and multipotent
- Can be passaged for weeks

- Express Nestin, Sox2, GFAP markers
- Serum-free, defined medium
- Rapid proliferation (48-72h doubling)

Embryonic Age Considerations:

- **E13-E15:** Optimal for neurosphere formation, high yield
- **E16-E18:** Still viable but lower neurosphere-forming capacity
- **Postnatal (P0-P7):** Can be used, but neurosphere formation decreases with age
- **Adult SVZ/SGZ:** Requires specialized protocols, lower yield

Safety & Animal Care

- Ensure proper IACUC approval for all animal procedures
- Timed pregnancies required (E13-E15 embryos)
- Follow institutional guidelines for pregnant animal handling
- Work in biosafety cabinet for sterile technique
- Wear appropriate PPE: lab coat, gloves, eye protection
- Dispose of animal tissues according to institutional policy

Required Materials

Reagents

- DMEM/F12 medium (1:1)
- N2 supplement (1× or 100×)
- B27 supplement (50× or 100×)
- Recombinant human EGF (20 ng/mL final)
- Recombinant human FGF-basic (20 ng/mL final)
- Penicillin/Streptomycin
- Chemical dissociation buffer (Sigma, Cat# C5914)
- Accutase or TrypLE (for passaging)
- PBS (phosphate buffered saline)

Equipment & Supplies

- Biological safety cabinet (BSC)
- 37°C CO_2 incubator
- 37°C water bath
- Centrifuge
- Dissection microscope
- Dissection tools (scissors, forceps)
- 10 cm Petri dishes (sterile, for dissection)
- Ultra-low attachment culture dishes (Corning)
- 15 mL conical tubes
- 1 mL pipette tips (for trituration)
- Hemocytometer or cell counter
- Ice bucket

Media Recipes

Incomplete Stem Cell Medium:

- DMEM/F12 (1:1)
- 1% Penicillin/Streptomycin
- Use for washing cells

Complete Stem Cell Medium:

- DMEM/F12 (1:1)
- 1% N2 supplement (if 100× stock)
- 2% B27 supplement (if 50× stock)
- 1% Pen/Strep
- 20 ng/mL EGF (add fresh)
- 20 ng/mL FGF-basic (add fresh)
- Sterile filter (0.22 µm)
- Store at 4°C, use within 2 weeks

Growth Factor Preparation

EGF Stock (100 µg/mL):

- Reconstitute in sterile PBS + 0.1% BSA
- Aliquot 50-100 µL, store at -20°C
- Dilute 1:5000 for 20 ng/mL final

FGF Stock (100 µg/mL):

- Reconstitute in sterile PBS + 0.1% BSA
- Aliquot 50-100 µL, store at -20°C
- Dilute 1:5000 for 20 ng/mL final

Animals

- **Preferred:** E13-E15 timed pregnant mice
- Can use any mouse strain
- Typical: 5-8 embryos per litter
- Expected yield: ~1-5 × 10^6 cells per embryo
- Each embryo yields 2 telencephalic vesicles

Recommended Dishes

- 6-well ultra-low attachment plate
- 10 cm ultra-low attachment dish
- T25 ultra-low attachment flask
- **Critical:** Must use ultra-low attachment surface
- Regular tissue culture dishes will cause cell adhesion

Neural Progenitor Isolation Protocol

> **Timing: ~2-3 hours from euthanasia to plating. Up to 5 vesicles can be processed per 15 mL tube.**

Step 1: Preparation

Mark Done

- Pre-warm water bath to 37°C
- Pre-warm incomplete stem cell medium (DMEM/F12 + Pen/Strep)
- Prepare ice bucket with 10 cm dish containing PBS or incomplete medium
- Prepare chemical dissociation buffer: 1 mL per 15 mL tube (up to 5 vesicles/tube)
- Keep dissociation buffer on ice until ready to use
- Sterilize dissection tools
- Prepare complete stem cell medium (add fresh EGF and FGF)

Step 2: Embryo Harvest

Mark Done

- Euthanize E13-E15 pregnant mouse per IACUC protocol
- Spray abdomen with 70% ethanol
- Make midline incision and expose uterine horns
- Remove entire uterus with embryos into dish with cold PBS

- Separate individual embryos from uterine tissue
- Remove embryos from embryonic sacs
- Decapitate embryos and collect heads in cold PBS on ice

Step 3: Brain Dissection

Mark Done

- Transfer embryonic head to fresh cold PBS in 10 cm dish
- Under dissection microscope, remove skin and skull carefully
- Expose the brain
- Carefully extract entire brain from skull
- Transfer brain to fresh cold medium

Step 4: Telencephalon Isolation

Mark Done

- Identify the two telencephalic vesicles (forebrain, bilateral structures)
- They are the largest, most anterior structures of the brain
- Using fine forceps, carefully separate telencephalic vesicles from rest of brain
- Remove meninges (thin membrane covering brain)
- Collect isolated vesicles in 15 mL tube with 1 mL cold chemical dissociation buffer
- Keep tube on ice during dissection
- Process up to 5 vesicles (from 2-3 embryos) per tube

Step 5: Chemical Dissociation

Mark Done

- After all vesicles collected, place tube in 37°C water bath
- Incubate for **10 minutes at 37°C**
- Gently swirl tube occasionally during incubation
- Tissue will soften but remain largely intact

Step 6: Neutralization and Washing

Mark Done

- Add equal volume (1 mL) of incomplete stem cell medium to tube
- Total volume now: 2 mL (1 mL buffer + 1 mL medium)

Step 7: Trituration

Mark Done

- Using 1 mL pipette tip, triturate tissue **15-20 times**
- Pipette gently at first, then more vigorously
- Avoid creating bubbles
- Continue until tissue is dissociated into single-cell suspension
- Solution should become cloudy and homogeneous
- No large tissue chunks should remain

Step 8: Centrifugation

Mark Done

- Centrifuge at **1,000 RPM for 4 minutes at 4°C**

- Cell pellet should be visible at bottom of tube
- Carefully aspirate supernatant without disturbing pellet

Step 9: Wash

Mark Done

- Resuspend pellet in 5-10 mL incomplete stem cell medium
- Centrifuge again at 1,000 RPM for 4 min at 4°C
- Aspirate supernatant
- This wash removes residual dissociation buffer

Step 10: Resuspension and Plating

Mark Done

- Resuspend cell pellet in complete stem cell medium (with EGF + FGF)
- Count cells using hemocytometer
- Expected yield: ~1-5 × 10^6 cells per embryo
- Plate at **1-5 × 10^4 cells/mL** in ultra-low attachment dishes
- Volume depends on dish size:
  - • 6-well plate: 2 mL per well
  - • 10 cm dish: 10 mL
  - • T25 flask: 5-10 mL
- Place in 37°C, 5% CO_2 incubator

Step 11: Neurosphere Formation

Mark Done

- Do not disturb cultures for first 24 hours
- Small neurospheres will start forming after 24-48 hours
- Neurospheres grow rapidly and reach 100-200 µm by 48-72 hours
- They appear as floating, refractile spheres
- Healthy neurospheres are round, bright, and smooth-edged

Neurosphere Maintenance and Passaging

> **Passaging Schedule**
>
> Neurospheres should be dissociated and passaged every 5-7 days to maintain healthy, proliferating progenitor cells. Do not allow neurospheres to grow too large (>300 µm) as cells in the center may become necrotic.

Feeding Neurospheres (Optional)

- Neurospheres can be maintained without feeding for 5-7 days
- If feeding: Add fresh complete medium with EGF/FGF at day 3-4
- Add 50% of original volume (e.g., if 2 mL, add 1 mL)

- Do not remove old medium - just add fresh

Passaging Protocol (Every 5-7 Days)

1. **Collect neurospheres:** Transfer suspension to 15 mL tube, allow spheres to settle by gravity (2-3 min)
2. **Remove medium:** Carefully aspirate supernatant, leaving neurospheres at bottom
3. **Wash (optional):** Add 5 mL incomplete medium, let settle, aspirate
4. **Dissociation:** Add Accutase or TrypLE (0.5-1 mL per 50-100 spheres)
5. **Incubate:** 5-10 minutes at 37°C with occasional gentle swirling
6. **Trituration:** Pipette 10-15 times with 1 mL tip to break up spheres into single cells
7. **Neutralize:** Add 5 mL incomplete medium
8. **Centrifuge:** 1,000 RPM for 4 min
9. **Resuspend:** In fresh complete medium with EGF/FGF
10. **Count and replate:** Plate at 1-5 × 10^4 cells/mL in ultra-low attachment dishes
11. **Passage number:** Keep track of passage number (P1, P2, etc.)

Freezing Neural Progenitors

- Dissociate neurospheres to single cells as in passaging protocol
- Resuspend at 1-5 × 10^6 cells/mL in freezing medium:
  - • 50% complete stem cell medium
  - • 40% FBS
  - • 10% DMSO
- Aliquot 1 mL per cryovial
- Freeze at -1°C/min to -80°C (use CoolCell or Mr. Frosty)
- Transfer to liquid nitrogen for long-term storage

Differentiation (Optional)

- **Neuronal differentiation:** Withdraw EGF/FGF, plate on laminin-coated dishes, add retinoic acid or BDNF
- **Astrocyte differentiation:** Add 1% FBS, withdraw growth factors, culture 7-14 days
- **Oligodendrocyte differentiation:** Add T3, PDGF, FGF, on laminin-coated dishes
- Confirm differentiation by immunocytochemistry for lineage markers

Neural Progenitor Characterization

• **Nestin:** Intermediate filament protein, neural stem cell marker

• **Sox2:** Transcription factor, maintains stemness

• **GFAP:** Can be expressed in neural stem cells

- **Ki67:** Proliferation marker, most cells should be positive
- **Negative for:** NeuN (mature neurons), O4 (oligodendrocytes), CD11b (microglia)

Troubleshooting

Problem	Possible Cause	Solution
No neurosphere formation	Wrong embryonic age Missing growth factors Cells plated on adherent dish	Use E13-E15 embryos Verify EGF and FGF were added Must use ultra-low attachment dishes Check growth factor activity
Very small neurospheres	Plating density too high Insufficient growth factors	Reduce plating density to 1-2 × 10^4/mL Add fresh EGF/FGF Use higher quality growth factors
Neurospheres too large/dark centers	Not passaged frequently enough Necrotic core forming	Passage every 5-7 days before spheres >300 μm Don't allow overgrowth Dissociate to single cells
Cells adhering to dish	Not using ultra-low attachment Serum contamination	Must use Corning ultra-low attachment dishes Ensure medium is serum-free Check for cross-contamination
Spheres falling apart	Over-trituration Excessive enzyme digestion	Reduce trituration force/number Shorten dissociation time Use gentler enzyme (Accutase)
Neurospheres not proliferating after passage	High passage number Over-digestion during passage Growth factor degraded	Use cells at P1-P10 for best proliferation Reduce enzyme exposure time Use fresh EGF/FGF aliquots

Problem	Possible Cause	Solution
		Store medium at 4°C, use within 2 weeks

Tips for Success

- Use E13-E15 embryos for best neurosphere formation
- Work quickly during dissection (keep tissue cold)
- Complete meninges removal to reduce contamination
- Do not over-digest tissue (10 min is usually sufficient)
- Must use ultra-low attachment dishes
- Add fresh EGF and FGF for every passage
- Passage regularly (don't let spheres get too large)
- Maintain passage records

Quality Control

- Monitor neurosphere size and number
- Healthy spheres: round, bright, smooth edges
- Verify Nestin and Sox2 expression
- Check proliferation (Ki67 staining)
- Test differentiation potential (tripotency)
- Use cells at low passage (P1-P10) for experiments
- Test for mycoplasma contamination