TECHNICAL MANUAL

In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™

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1.0 NeuroCult™ Mouse & Rat Ordering Information

PRODUCT NAME	CATALOG #	SIZE	KIT COMPONENTS
NeuroCult™ Basal Medium (Mouse & Rat)	05700	450 mL	Not applicable
NeuroCult™ Proliferation Supplement (Mouse & Rat)	05701	50 mL	Not applicable
NeuroCult™ Differentiation Supplement (Mouse & Rat)	05703	50 mL	Not applicable
NeuroCult™ Proliferation Kit (Mouse & Rat)	05702	1 Kit	• NeuroCult™ Basal Medium (Mouse & Rat) • NeuroCult™ Proliferation Supplement (Mouse & Rat)
NeuroCult™ Differentiation Kit (Mouse & Rat)	05704	1 Kit	• NeuroCult™ Basal Medium (Mouse & Rat) • NeuroCult™ Differentiation Supplement (Mouse & Rat)

- For proliferation and differentiation of **mouse** neural stem and progenitor cells, refer to sections 2.0 - 8.0.
- For proliferation and differentiation of **rat** neural stem and progenitor cells, refer to sections 9.0 - 14.0.

2.0 Mouse: Required Materials and Equipment

2.1 Dissociation of Mouse Central Nervous System (CNS) Tissue

- Phosphate-buffered saline (PBS) containing 2% glucose (required for collecting tissue)
- Trypan Blue (Catalog #07050)
- NeuroCult™ Enzymatic Dissociation Kit for Adult CNS Tissue (Mouse & Rat; Catalog #05715) [optional]

2.2 Proliferation of Mouse Neural Stem and Progenitor Cells

- Human Recombinant EGF (100 µg, Catalog #78006.1)
- Human Recombinant bFGF (10 µg, Catalog #78003.1) [only needed when using ADULT mouse CNS cells]
- Heparin Solution (Catalog #07980; only needed when using ADULT mouse CNS cells)
- Bovine serum albumin (BSA) or human serum albumin (HSA); required for reconstitution of EGF and bFGF
- Poly-D-Lysine (PDL; Sigma Catalog #P7280) [optional; only needed for adherent cultures]
- Laminin (Sigma Catalog #L2020) [optional; only needed for adherent cultures]
- NeuroCult™ Chemical Dissociation Kit (Catalog #05707) [optional; for passaging cells]
- ACCUTASE™ (Catalog #07920) [optional; for passaging cells]
- Trypan Blue (Catalog #07050)

Table 1. Recommended Cultureware for Proliferation (Neurosphere and Adherent Monolayer Methods)

CULTUREWARE	RECOMMENDED SUPPLIERS
6-well plate	Costar® 6-Well Flat-Bottom Plate, Tissue Culture-Treated (STEMCELL Catalog #38015)
T-25 cm^2 flask	Nunc™ EasYFlask™ (Thermo Fisher Catalog #156367 or VWR Catalog #15708-130) Corning® Cell Culture Flask (Corning Catalog #430639 or VWR Catalog #29186-010)
T-75 cm^2 flask	Corning® Cell Culture Flask (Corning Catalog #3276 or Fisher Scientific Catalog #07-200-66)
T-162 cm^2 flask	Corning® Costar® Cell Culture Flask (Corning Catalog #3151 or Fisher Scientific Catalog #07-200-64)

2.3 Differentiation of Mouse Neural Stem and Progenitor Cells

- 12 mm round glass coverslips (Carolina Biological Catalog #633029)
- Adhesive substrates recommended for differentiation of **embryonic** mouse CNS cells:
  - Poly-L-Ornithine (PLO) Solution (Sigma Catalog #P4957)
  - Poly-D-Lysine (PDL; Sigma Catalog #P7280)
  - Laminin (Sigma Catalog #L2020)

- Adhesive substrate recommended for differentiation of **adult** mouse CNS cells:
  - Corning® Matrigel® hESC-Qualified Matrix (Corning Catalog #354277)

    OR
  - Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning Catalog #354230)
- Trypan Blue (Catalog #07050)

Table 2. Recommended Cultureware for Differentiation

CULTUREWARE	RECOMMENDED SUPPLIERS
24-well plate	Costar® 24-Well Flat-Bottom Plate, Tissue Culture-Treated (STEMCELL Catalog #38017)
8-well culture slide	Corning® BioCoat™ Poly-D-Lysine 8-Well Culture Slides (Corning Catalog #354632) Corning® BioCoat™ Poly-D-Lysine/Laminin 8-Well Culture Slides (Corning Catalog #354688)

2.4 Immunolabeling Differentiated Cells

- D-PBS (Without Ca++ or Mg++) (PBS; Catalog #37350)
- 4% Paraformaldehyde (in PBS pH 7.2; Sigma Catalog #P6148)
- Triton™ X-100 (Sigma Catalog #T-9284)
- Mounting medium (e.g. FluorSave™ Reagent, EMD Millipore Catalog #345789)

2.5 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Benchtop centrifuge
- Vortex
- Pipette-aid
- Pipettors: 10 µL (Catalog #38061), 200 µL (Catalog #38059), and 1 mL (Catalog #38058) with sterile disposable plastic pipette tips
- 15 mL polypropylene conical tubes (Catalog #38009)

  OR

  14 mL polystyrene round-bottom tubes, 17 x 95 mm (Catalog #38008)
- 50 mL polypropylene conical tubes (Catalog #38010)
- Hemocytometer
- Forceps for use in moving slides used in differentiation
- Routine light microscope for hemocytometer cell counts
- Inverted microscope with total magnification of approximately 20 - 30X, 80X, and 125X

3.0 Mouse: Preparation of Reagents and Cultureware

3.1 For Proliferation of Neural Stem and Progenitor Cells

3.1.1 EGF Stock Solution (10 µg/mL)

1. Add 1 mL sterile water containing at least 0.1% BSA to EGF (100 µg/vial) by pipetting the solution down the sides of the vial. Do not vortex.

   Note: If not used immediately, store 100 µg/mL EGF at -20°C to -80°C for up to 6 months.

2. Dilute EGF (100 µg/mL) 1 in 10 with sterile water containing at least 0.1% BSA (final concentration 10 µg/mL).

   Note: If not used immediately, aliquot stock solution (0.1 - 0.3 mL) and store at -20°C. Do not freeze/thaw each aliquot more than 3 times.

3.1.2 bFGF Stock Solution (10 µg/mL)

*Note: bFGF is only needed when culturing **adult** mouse CNS cells.*

1. Add 1 mL sterile water containing at least 0.1% BSA to bFGF (10 µg/vial) by pipetting the solution down the sides of the vial. Do not vortex.

   Note: If not used immediately, aliquot stock solution (0.1 - 0.3 mL) and store at -20°C. Do not freeze/thaw each aliquot more than 3 times.

3.1.3 NeuroCult™ Proliferation Media

1. Thaw one 50 mL bottle of NeuroCult™ Proliferation Supplement at 2 - 8°C overnight or at 37°C for 1 - 2 hours.

   Note: If not used immediately, aliquot into 10 mL volumes and store at -20°C. Once aliquots are thawed, use immediately. Do not re-freeze. A white precipitate may form after storage at -20°C. The precipitate will disappear after complete thawing at 37°C and mixing the contents.

   Note: If the supplement thawed during shipping, immediately aliquot and store at -20°C. If the supplement remained cool (not more than ~10°C), performance should be unaffected.

2. Add the entire volume (50 mL) of NeuroCult™ Proliferation Supplement to one bottle (450 mL) of NeuroCult™ Basal Medium.

   OR

   Add 1 mL NeuroCult™ Proliferation Supplement to every 9 mL NeuroCult™ Basal Medium.

3. Mix thoroughly. **NeuroCult™ Proliferation Medium <u>without cytokines</u>** is now ready for use.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month. Avoid repeated exposure of medium to room temperature and light.

   Note: NeuroCult™ Proliferation Medium without cytokines is only used in very specific steps in the procedure, e.g. when resuspending and washing cell suspensions. Do not use this cytokine-free medium for cell culture.

4. Different cytokine combinations are recommended when culturing cells obtained from embryonic or adult mouse tissue, as follows:

   For culturing cells from EMBRYONIC mouse CNS:

   To each 10 mL of NeuroCult™ Proliferation Medium without cytokines (prepared in step 3), add the following:

   - 20 µL of 10 µg/mL EGF (to give a final concentration of 20 ng/mL EGF)

   This will be referred to as **Complete Embryonic NeuroCult™ Proliferation Medium**.

   Note: If not used immediately, store Complete Embryonic NeuroCult™ Proliferation Medium at 2 - 8°C for up to 2 weeks.

   For culturing cells from ADULT mouse CNS:

   To each 10 mL of NeuroCult™ Proliferation Medium without cytokines (prepared in step 3), add the following:

   - 20 µL of 10 µg/mL EGF (to give a final concentration of 20 ng/mL EGF)
   - 10 µL of 10 µg/mL bFGF (to give a final concentration of 10 ng/mL bFGF)
   - 10 µL of Heparin Solution (to give a final concentration of 0.0002% Heparin (w/v); equals 2 µg/mL)

   This will be referred to as **Complete Adult NeuroCult™ Proliferation Medium**.

   Note: If not used immediately, store Complete Adult NeuroCult™ Proliferation Medium at 2 - 8°C for up to 2 weeks.

3.1.4 Coating Cultureware for Adherent Monolayer Cultures

To culture neural stem and progenitor cells as adherent monolayers, an appropriate substrate is required to coat the surface of the cultureware. By coating the surface of the cultureware with poly-D-lysine (PDL), laminin, poly-L-ornithine (PLO), or combinations of these substrates, cell-to-substrate attachment is promoted, as opposed to the cell-to-cell attachment and aggregation which results in neurosphere formation in the absence of an appropriate substrate.

The following instructions are for preparation of PDL and laminin stock solutions (sections A & B), PDL/laminin-coated cultureware (section C), and laminin-only coated cultureware (section D).

A. Preparation of 100 µg/mL PDL Stock Solution

1. Dissolve 5 mg poly-D-lysine in 50 mL sterile water.
2. Aliquot solution in polypropylene vials and store at 2 - 8°C.

B. Preparation of 10 µg/mL Laminin Stock Solution

1. Thaw laminin at 2 - 8°C.
2. Prepare a 10 µg/mL working solution of laminin by diluting the laminin in sterile PBS or water (the amount prepared should correspond to the amount needed for immediate use).
3. Store the remaining laminin (which has not been diluted) in appropriately sized aliquots at -20°C.

C. Preparation of PDL/Laminin-Coated Cultureware

1. Dilute the 100 µg/mL PDL stock solution with sterile water to a final concentration of 10 µg/mL.
2. Dispense the appropriate volume of 10 µg/mL PDL solution for the chosen cultureware as indicated in Table 3.

Table 3. Substrate Volumes for Coating Cultureware

CULTUREWARE	VOLUME OF SUBSTRATE SOLUTION
6-well plate	1 - 2 mL/well
T-25 cm^2 flask	3 mL

3. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight (~20 hours).
4. Wash each well/flask with sterile PBS according to the recommended volume in Table 4. Remove as much of the PBS as possible.

Table 4. PBS Wash Volumes

CULTUREWARE	VOLUME OF PBS WASH
6-well plate	1 - 2 mL/well
T-25 cm^2 flask	5 mL

5. Dispense 10 µg/mL laminin stock solution at the volume indicated in Table 3.
6. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight.
7. Wash each well/flask with sterile PBS according to the recommended volumes in Table 4.
   Note: Only remove the PBS when ready to plate the cells. Do not let the coated plates dry completely.
8. The substrate-coated cultureware is ready for use. If not used immediately, wrap the plate with Parafilm®, do not remove PBS from the wells, and store the plate at 2 - 8ºC for up to 2 weeks.
9. Proceed to section 6.0 for plating cells for adherent monolayer cultures.

D. Preparation of Laminin-Only Coated Cultureware

1. Dispense 10 µg/mL laminin stock solution at the volume indicated in Table 3.
2. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight.
3. Wash each well/flask with sterile PBS according to the recommended volume in Table 4.
   Note: Only remove the PBS when ready to plate the cells. Do not let the coated plates dry completely.
4. The substrate-coated cultureware is ready for use. If not used immediately, wrap the plate with Parafilm®, do not remove PBS from the wells, and store the plate at 2 - 8ºC for up to 2 weeks.
5. Proceed to section 6.0 for plating cells for adherent monolayer cultures.

3.2 For Differentiation of Neural Stem and Progenitor Cells

3.2.1 Complete NeuroCult™ Differentiation Medium

1. Thaw one 50 mL bottle of NeuroCult™ Differentiation Supplement at 2 - 8°C overnight or at 37°C for 1 - 2 hours.

   Note: If not used immediately, aliquot into 10 mL volumes and store at -20°C. Once aliquots are thawed, use immediately. Do not re-freeze. A white precipitate may form after storage at -20°C. The precipitate will disappear after complete thawing at 37°C and mixing the contents.

2. Add the entire volume (50 mL) of NeuroCult™ Differentiation Supplement to 1 bottle (450 mL) of NeuroCult™ Basal Medium.

   OR

   Add 1 mL NeuroCult™ Differentiation Supplement to every 9 mL NeuroCult™ Basal Medium (1 in 10 dilution).

3. Mix thoroughly. Complete NeuroCult™ Differentiation Medium is now ready for use.

   Note: If not used immediately, store Complete NeuroCult™ Differentiation Medium at 2 - 8°C for up to 1 month.

3.2.2 Coating Coverslips for Differentiation Cultures

If using round glass coverslips for immunocytochemistry, ensure that the chosen coverslips fit easily in and out of the wells of the plate used for culturing the cells. Sterilize coverslips by autoclaving prior to coating.

- When differentiating cells obtained from **embryonic** mouse CNS tissue, use PLO/laminin-coated or PDL/laminin-coated glass coverslips (section A).
- When differentiating cells obtained from **adult** mouse CNS tissue, use Matrigel®-coated glass coverslips (section B).

A. Preparation of PLO/Laminin- or PDL/Laminin-Coated Coverslips

PLO/laminin- or PDL/laminin-coated coverslips are recommended when differentiating cells obtained from **embryonic** mouse CNS tissue.

1. Preparation of PLO or PDL stock solutions

Preparation of 15 µg/mL PLO Stock Solution

Add 1.5 mL Poly-L-Ornithine Solution (0.01%) to 8.5 mL sterile PBS.

OR

Preparation of 100 µg/mL PDL Stock Solution

a. Dissolve 5 mg PDL in 50 mL sterile water.
b. Aliquot solution in polypropylene vials and store at 2 - 8°C.

2. Preparation of 10 µg/mL laminin stock solution

a. Thaw laminin at 2 - 8°C, to prevent laminin from gelling.
b. Prepare a 10 µg/mL working solution of laminin by diluting the laminin in sterile PBS or sterile water (the amount prepared should correspond to the amount needed for immediate use).
c. Store the remaining laminin (which has not been diluted) in appropriately sized aliquots at -20°C.

3. Preparation of coated coverslips

a. Using sterile forceps, transfer one sterile glass coverslip per well of a 24-well plate.

For PLO:

Dispense 0.5 - 1 mL of the 15 µg/mL PLO stock solution into each well containing a coverslip.

For PDL:

Dilute the 100 µg/mL PDL stock solution with sterile water to a final concentration of 10 µg/mL. Dispense 0.5 - 1 mL of the 10 µg/mL PDL solution into each well containing a coverslip.

Note: Ensure that the coverslips are completely submerged in the PLO or PDL solution. If necessary, use a plastic disposable pipette tip to push the coverslip to the bottom of the well.

b. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight (~20 hours).

c. Wash each well with 2 x 1 mL sterile PBS. Remove as much of the PBS as possible.

d. Dispense 0.5 - 1 mL of the 10 µg/mL laminin stock solution into each well containing a coverslip.

e. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight.

f. Remove the solution from each well. Wash each well 3 times for 15 minutes each with 1 mL sterile PBS to remove any residual laminin solution.

Note: Only remove the PBS when ready to plate the cells. Do not let the coated plates dry completely.

g. The substrate-coated coverslips are ready for use. If not used immediately, wrap the plate with Parafilm®, do not remove PBS from the wells, and store the plate at 2 - 8ºC for up to 2 weeks.

B. Matrigel®-Coated Coverslips

Matrigel®-coated coverslips are recommended when differentiating cells obtained from **adult** mouse CNS tissue. Use either Corning Catalog #354277 (section 1) or Corning Catalog #354230 (section 2).

1. Corning® Matrigel® hESC-Qualified Matrix (Corning Catalog #354277)

a. Thaw Matrigel® at 2 - 8°C until it liquefies.

Note: To prevent gelation, Matrigel® must be kept cold (keep on ice or at 2 - 8˚C).

b. Refer to the Matrigel® Certificate of Analysis for the recommended aliquot size (“Dilution Factor”) to prepare 25 mL of diluted matrix.

c. Aliquot the entire bottle of Matrigel® into 2 mL screw cap tubes. Set aside a sufficient number of aliquots for experimental use and store the remaining unused aliquots at -20°C.

Note: One Matrigel® aliquot generates enough solution to coat one or two 24-well plates (depending on whether 0.5 or 1 mL of solution is used to coat each well).

d. Add 25 mL of **cold** NeuroCult™ Proliferation Medium without cytokines (section 3.1.1 steps 1 - 3) to a 50 mL polypropylene tube. Keep on ice.

e. Transfer thawed Matrigel® to the tube prepared in step d. Pipette up and down to mix. The vial may be washed with cold medium if desired.

f. Using sterile forceps, transfer one sterile glass coverslip per well of a 24-well plate.

g. Add 0.5 - 1 mL of diluted Matrigel® to each well containing a coverslip.

h. Incubate at room temperature (15 - 25°C) for 2 hours or overnight at 2 - 8°C.

i. Remove the Matrigel® solution when ready to use the coated coverslips.

Note: Cells can be plated directly onto the Matrigel®-coated coverslips without the need for a wash step.

2. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning Catalog #354230)

a. Thaw Matrigel® at 2 - 8°C until it liquefies.

Note: To prevent gelation, Matrigel® must be kept cold (keep on ice or at 2 - 8˚C).

b. The protein concentration of each lot of Matrigel® varies. Refer to the Matrigel® Certificate of Analysis to determine the appropriate volume of Matrigel® required to prepare 2 mg aliquots.

c. Aliquot the entire bottle of thawed Matrigel® into 2 mg aliquots in 2 mL screw cap tubes. Set aside a sufficient number of 2 mg aliquots for experimental use and store the remaining unused aliquots at -20°C.

Note: One 2 mg Matrigel® aliquot generates enough solution to coat one or two 24-well plates (depending on whether 0.5 or 1 mL of solution is used to coat each well).

d. Add 23 mL of NeuroCult™ Proliferation Medium without cytokines (section 3.1.1 steps 1 - 3) into a 50 mL polypropylene tube.

e. Add 1 mL of **cold** NeuroCult™ Proliferation Medium without cytokines to the tube containing 2 mg Matrigel® and pipette up and down to mix the solution.

f. Transfer the diluted Matrigel® solution to the 50 mL tube containing 23 mL NeuroCult™ Proliferation Medium without cytokines and pipette up and down gently to mix.

g. Using sterile forceps, transfer one sterile glass coverslip per well of a 24-well plate.

h. Add 0.5 - 1 mL of medium + Matrigel® (prepared in step e) to each well containing a coverslip.

i. Incubate at room temperature (15 - 25°C) for 2 hours or overnight at 2 - 8°C.

j. Remove the Matrigel® solution when ready to use the coated coverslips.

Note: Cells can be plated directly onto the Matrigel®-coated coverslips without the need for a wash step.

4.0 Mouse: Dissociation of CNS Tissue

4.1 Dissociation of Embryonic Day 14 (E14) Mouse CNS Tissue

1. Dissect embryos at gestational day E14, where E0 is the day a gestational plug forms.

   Note: The number of embryos obtained from one pregnant mouse varies from 8 - 12 embryos. The protocol below can be used to process up to 20 embryos.
2. Remove the entire brain from the embryos and transfer to a 35 mm dish containing **cold** PBS containing 2% glucose [Refer to Cell Biology: A Laboratory Handbook. ed. Julio E. Cells. 1998. Volume 1, p149 for additional information].
3. Dissect out desired brain regions and place in **cold** PBS containing 2% glucose on ice.
4. When dissections are complete, transfer all tissue in PBS containing 2% glucose into a 15 mL conical tube or 14 mL round-bottom tube.
5. Allow tissue to settle and pipette off supernatant. Resuspend tissue in 1 mL Complete Embryonic NeuroCult™ Proliferation Medium (see section 3.1.3 step 4).
6. Using a disposable plastic tip attached to a 1 mL pipettor set at 0.9 mL, triturate the tissue approximately 5 times. If undissociated tissue remains, allow the clumps to settle for 1 - 2 minutes. Pipette off the supernatant containing the single cells into a new 15 mL conical tube. Discard undissociated tissue.

   Note: Triturate to create a single-cell suspension, but be careful to not create air bubbles. The majority of the tissue will be dissociated in this step. Do not excessively triturate, as this will result in increased cell death.
7. Add 10 mL of Complete Embryonic NeuroCult™ Proliferation Medium to the single-cell suspension.
8. Centrifuge at 150 x *g* for 5 minutes. Remove and discard supernatant.
9. Resuspend cells with a brief trituration (2 times) with a disposable plastic pipette tip in 1 mL Complete Embryonic NeuroCult™ Proliferation Medium. Add an additional 2 mL of Complete Embryonic NeuroCult™ Proliferation Medium.
10. Filter the cell suspension through a 37 µm strainer (e.g. Catalog #27215).
11. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.
12. Proceed to sections 5.0 and 6.0 for plating cells for neurosphere and adherent monolayer cultures, respectively.

4.2 Dissociation of Adult Mouse CNS Tissue

NeuroCult™ Enzymatic Dissociation Kit for Adult CNS Tissue (Mouse & Rat; Catalog #05715) effectively dissociates adult mouse CNS tissue to obtain a cell suspension with high cell viability. For complete instructions, refer to the Technical Manual: Enzymatic Dissociation of Adult Mouse and Rat CNS Tissue Using NeuroCult™ Enzymatic Dissociation Kit, available at www.stemcell.com or contact us to request a copy.

1. Once a single-cell suspension has been obtained, count viable cells using Trypan Blue and a hemocytometer.
2. Proceed to sections 5.0 and 6.0 for details on plating cells for neurosphere and adherent monolayer cultures, respectively.

5.0 Mouse: Expansion of Neural Stem and Progenitor Cells in Neurosphere Cultures

5.1 Initial Plating of Primary Mouse CNS Cells in Neurosphere Cultures

1. Plate **embryonic** CNS-derived cells in Complete Embryonic NeuroCult™ Proliferation Medium as indicated in Table 5.

Table 5. Embryonic Mouse CNS-Derived Cell Plating Conditions for Neurosphere Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
T-25 cm^2 flask	10 mL	8 x 10^4 viable cells/cm^2	2 x 10^6 cells
T-75 cm^2 flask	20 mL	7 x 10^4 viable cells/cm^2	5 x 10^6 cells
T-162 cm^2 flask	40 mL	5 x 10^4 viable cells/cm^2	8 x 10^6 cells

OR

Plate **adult** CNS-derived cells in Complete Adult NeuroCult™ Proliferation Medium as indicated in Table 6.

Table 6. Adult Mouse Subventricular Zone-Derived Cell Plating Conditions for Neurosphere Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
6-well plate	3 mL	2 x 10^4 viable cells/cm^2	1.9 x 10^5 cells
T-25 cm^2 flask	10 mL	2 x 10^4 viable cells/cm^2	5 x 10^5 cells

2. Incubate at 37°C and 5% CO_2 in a humidified incubator.
3. Proceed to section 5.2 for passaging neurospheres.

5.2 Harvesting Cells for Passaging Neurosphere Cultures

Neurospheres should be passaged when they reach 100 - 150 µm in diameter (typically occurs 5 - 8 days after plating). Passaging should also be performed before the cells reach high densities and prior to the growth medium becoming acidic (turns orange/yellow in color). If medium does turn orange/yellow in color before the neurospheres have reached 100 - 150 µm in diameter, perform a half-medium change.

Do not let the neurospheres grow too large (> 200 µm in diameter); the cells within the core of large neurospheres lack appropriate gas and nutrient/waste exchange, and become necrotic.

1. Harvest and collect the entire cell suspension from the culture into a 50 mL conical tube (if using a T-75 cm^2 or T-162 cm^2 flask), or a 15 mL conical tube (if using a T-25 cm^2 flask). If neurospheres are attached to the culture flask, tap the culture flask or dispense a stream of medium across the attached cells to detach them.
2. Centrifuge cells at 90 x *g* for 5 minutes.
3. Proceed to section 5.3 and select the desired dissociation method.

5.3 Dissociation of Neurospheres

Dissociate neurospheres using chemical dissociation (section 5.3.1), ACCUTASE™ enzymatic dissociation (section 5.3.2), or mechanical dissociation (section 5.3.3).

5.3.1 Chemical Dissociation

Dissociation of neurospheres with the NeuroCult™ Chemical Dissociation Kit results in high cell viability. For more information on how to chemically dissociate neurospheres, refer to the Technical Manual: Chemical Dissociation of Neurospheres Derived from Embryonic and Adult Mouse CNS Using the NeuroCult™ Chemical Dissociation Kit, available at www.stemcell.com or contact us to request a copy.

After obtaining chemically dissociating neurospheres and obtaining a single-cell suspension, count viable cells using Trypan Blue and a hemocytometer. Proceed to section 5.4 for replating cells.

5.3.2 ACCUTASE™ Enzymatic Dissociation

ACCUTASE™ contains both proteolytic and collagenolytic enzymes and is useful for the routine dissociation of cells.

1. Thaw ACCUTASE™ at 2 - 8 °C overnight, in a vessel of cool water, or at room temperature (15 - 25°C) only until thawed. Do not thaw at 37°C.
2. Following centrifugation of cells (section 5.2 step 2), remove and discard the supernatant, leaving behind the cell pellet.
3. Pre-wet the disposable pipette tip with the appropriate (i.e. embryonic or adult) Complete NeuroCult™ Proliferation Medium before dissociating cells, to prevent the cells from sticking to the walls of the pipette tip.
4. Add the appropriate volume of ACCUTASE™ to the cell pellet as indicated below:
   - T-25 cm^2 flask : 200 µL per cell pellet harvested
   - T-75 cm^2 flask: 500 µL per cell pellet harvested
   - T-162 cm^2 flask: 1 mL per cell pellet harvested
5. Incubate at room temperature (15 - 25°C) or at 37°C for 5 minutes. At the mid-point of the incubation, mix by gently shaking the tube, to ensure the cell suspension is well mixed.
6. After 5 minutes, determine by eye if there are any undissociated neurospheres or aggregates remaining. If clumps remain, perform a gentle trituration step using a pipettor. For cells harvested from a T-25 cm^2 flask, use a 200 µL pipettor set to 180 µL. For cells harvested from a T-75 cm^2 or T-162 cm^2 flask, use a 1 mL pipettor set to 490 µL or 990 µL, respectively.
7. Wash the cells with NeuroCult™ Proliferation Medium without cytokines (cytokine addition is not required at this stage).
8. Centrifuge at 150 x *g* for 5 minutes. Discard the supernatant.
9. Resuspend the cells in ~0.5 mL of Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium.
10. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer, then proceed to section 5.4 for replating cells.

5.3.3 Mechanical Dissociation

1. Following centrifugation of cells (section 5.2 step 2), remove and discard the supernatant.
2. For cultures obtained from **T-25 cm^2 flasks**:
   - Resuspend the cell pellet in a maximum of 0.2 mL Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium (depending on the cell source).
   - If more than one tube was used to harvest the cultures, resuspend each cell pellet in a small volume (e.g. 200 µL) and pool all cell suspensions.

   For cultures obtained from **T-75 cm^2** or **T-162 cm^2 flasks**:
   - Resuspend the cell pellet in 0.5 mL of Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium (depending on the cell source).
   - If a 50 mL tube was used in the initial harvest (section 5.2 step 1), transfer the cell suspension to a 15 mL tube (for a more efficient dissociation). If more than 1 tube was used to harvest the cultures, resuspend each cell pellet in a small volume (e.g. 200 µL) and pool all cell suspensions.
3. Triturate neurospheres by pipetting up and down with a 200 µL pipettor set at ~180 µL until a single- cell suspension is achieved (approximately 20 - 30 times). Triturate vigorously, but do not introduce air bubbles into the cell suspension.
4. If undissociated neurospheres remain, allow to settle for 1 - 2 minutes and pipette off supernatant containing single cells into a new tube. Repeat trituration if necessary, as follows:
   - Add 200 µL Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium to the remaining undissociated neurospheres and continue to triturate as indicated in step 3.
5. Once a single-cell suspension has been obtained, pool all cell suspensions.
6. Centrifuge at 150 x *g* for 5 minutes.
7. Remove supernatant and resuspend the cells by brief trituration in an appropriate volume (e.g. 1 mL) of Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium as necessary.
8. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer. Proceed to section 5.4 for replating cells.

5.4 Replating Cells for Neurosphere Cultures

1. Plate **embryonic** CNS-derived cells in Complete Embryonic NeuroCult™ Proliferation Medium as indicated in Table 7.

Table 7. Plating Conditions for Neurosphere Cultures of EMBRYONIC CNS-Derived Cells

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
T-25 cm^2 flask	10 mL	2 x 10^4 viable cells/cm^2	5 x 10^5 cells
T-75 cm^2 flask	20 mL	1.7 x 10^4 viable cells/cm^2	1.25 x 10^6 cells
T-162 cm^2 flask	40 mL	1.2 x 10^4 viable cells/cm^2	2 x 10^6 cells

OR

Plate adult **CNS-derived cells in Complete Adult NeuroCult™ Proliferation Medium as indicated in**

Table 8.

Table 8. Plating Conditions for Neurosphere Cultures of ADULT Mouse Subventricular Zone-Derived Cells

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
T-25 cm^2 flask	10 mL	4 x 10^3 viable cells/cm^2	1 x 10^5 cells
T-75 cm^2 flask	20 mL	3 x 10^3 viable cells/cm^2	2.5 x 10^5 cells
T-162 cm^2 flask	40 mL	2.5 x 10^3 viable cells/cm^2	4 x 10^5 cells

2. Incubate at 37°C and 5% CO_2 in a humidified incubator.

6.0 Mouse: Expansion of Neural Stem and Progenitor Cells in Adherent Monolayer Cultures

6.1 Initial Plating of Primary Mouse CNS Cells in Adherent Monolayer Cultures

1. Prepare single-cell suspensions of embryonic or adult mouse CNS cells according to section 4.0.
2. Plate **embryonic** mouse CNS-derived cells in Complete Embryonic NeuroCult™ Proliferation Medium into **cultureware coated with PDL/laminin or laminin** as indicated in Table 9.

Table 9. Embryonic CNS-Derived Cell Plating Conditions for Adherent Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
6-well plate	3 mL/well	8 x 10^4 viable cells/cm^2	7.6 x 10^5 cells
T-25 cm^2 flask	10 mL	8 x 10^4 viable cells/cm^2	2 x 10^6 cells

OR

Plate **adult** CNS-derived cells in Complete Adult NeuroCult™ Proliferation Medium into **cultureware coated with PDL/laminin or laminin** as indicated in Table 10.

Table 10. Adult CNS-Derived Cell Plating Conditions for Adherent Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
6-well plate	3 mL/well	2 x 10^4 viable cells/cm^2	1.9 x 10^5 cells
T-25 cm^2 flask	10 mL	2 x 10^4 viable cells/cm^2	5 x 10^5 cells

3. Incubate at 37°C and 5% CO_2 in a humidified incubator.

   Note: In the presence of a substrate, neural stem and progenitor cells will adhere to the substrate-coated cultureware within 24 hours. The attached cells show a flattened morphology and are mostly bipolar. Refer to section 8.2 for images of adherent monolayer cultures.

6.2 Passaging Cells from Adherent Monolayer Cultures

Cultures should be passaged when they reach 60 - 80% confluence.

Note: Before harvesting the cells, it is important to prepare the required number of coated wells or flasks needed for subculture.

The procedure outlined below uses ACCUTASE™ to dissociate the cells. ACCUTASE™ dissociation results in high cell viability and ability of the dissociated cells to initiate new adherent cultures for multiple passages.

1. Once the culture is 60 - 80% confluent and is ready for passaging, use a pipettor to remove the medium from the culture vessel.
2. Add PBS to wash the cells as follows:
   - One well of a 6-well plate: 3 mL PBS
   - T-25 cm^2 flask: 10 mL PBS
3. Swirl the culture plate or flask gently, then remove PBS and discard.

4. Add ACCUTASE™ as follows:
   - One well of a 6-well plate: 0.5 mL ACCUTASE™
   - T-25 cm^2 flask: 1 mL ACCUTASE™
5. Incubate at room temperature (15 - 25°C) or at 37°C for 5 minutes.
6. Observe the culture to determine whether the cells are starting to detach and detachment is complete. If cells have not completely detached after 5 minutes, dispense a stream of medium across the surface of the cultureware to detach the cells.
7. Add Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium (choice dependent on cell source; see section 3.1.3) to the detached cells using a disposable pipette as follows:
   - One well of a 6-well plate: 2 mL medium
   - T-25 cm^2 flask: 5 mL medium
8. Using the same pipette, resuspend the detached cells by pipetting the cell/medium suspension up and down 2 - 3 times.
9. Collect the cells and place in a new sterile 15 mL conical tube. If cells remain in the vessel, add a small volume of Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium and repeat the procedure to collect the remaining cells.
10. Centrifuge at 110 x *g* for 5 minutes.
11. Remove the supernatant and resuspend cells in a maximum of 200 µL Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium with a plastic disposable pipette tip attached to a 200 µL pipettor set at ~180 µL, pipetting until a single-cell suspension is achieved.
12. Resuspend cells in an appropriate volume (approximately 0.5 - 1 mL) of Complete Embryonic or Complete Adult NeuroCult™ Proliferation Medium.
13. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer. Proceed to section 6.3 for replating.

6.3 Replating Cells for Adherent Monolayer Cultures

1. Plate **embryonic** mouse CNS-derived cells in Complete Embryonic NeuroCult™ Proliferation Medium into **cultureware coated with PDL/laminin or laminin** as indicated in Table 11.

Table 11. EMBRYONIC CNS-Derived Cell Plating Conditions for Adherent Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
6-well plate	3 mL/well	2 x 10^4 viable cells/cm^2	1.9 x 10^5 cells
T-25 cm^2 flask	10 mL	2 x 10^4 viable cells/cm^2	5 x 10^5 cells

OR

Plate **adult** CNS-derived cells in Complete Adult NeuroCult™ Proliferation Medium into **cultureware coated with PDL/laminin or laminin** as indicated in Table 12.

Table 12. ADULT CNS-Derived Cell Plating Conditions for Adherent Cultures

CULTUREWARE	VOLUME OF MEDIUM	CELL DENSITY	TOTAL # OF CELLS
6-well plate	3 mL/well	8 x 10^3 viable cells/cm^2	7.6 x 10^4 cells
T-25 cm^2 flask	10 mL	8 x 10^3 viable cells/cm^2	2 x 10^5 cells

2. Incubate at 37°C and 5% CO_2 in a humidified incubator.

Note: This procedure can be repeated for multiple passages.

7.0 Mouse: Differentiation of Neural Stem and Progenitor Cells

In the presence of cytokines (EGF and bFGF), neural stem cells remain in a relatively undifferentiated state. Upon removal of cytokines and the addition of a small amount of serum, differentiation is induced to neurons, astrocytes, and oligodendrocytes. Alternatively, specific cytokines can be used to direct differentiation to specific cell lineages. Refer to section 7.4 for an alternative differentiation method, making use of bFGF stimulation, to obtain increased numbers of neurons.

For representative images, refer to section 8.1 (neurosphere cultures) and 8.2 (adherent monolayer cultures).

7.1 Harvesting Cells from Neurosphere Cultures for Differentiation

The procedure below has been optimized for cells cultured in a T-25 cm^2 flask. If using other cultureware, adjust volumes accordingly.

1. Harvest neurospheres when they reach 100 - 150 µm in diameter. After plating, this typically takes 5 - 7 days for embryonic CNS-derived cells, whereas for adult CNS-derived cells, this typically takes 7 - 8 days for primary cultures and 5 - 7 days for passaged cells.
2. Collect neurospheres and place in a 15 mL conical tube. If some cells remain attached to the flask, detach them by dispensing a stream of medium across the attached cells.
3. Centrifuge at 90 x *g* for 5 minutes. Remove supernatant and discard.
4. Wash the cells (to remove the cytokines) by gently resuspending the cell pellet in 10 mL Complete NeuroCult™ Differentiation Medium.
5. Centrifuge at 90 x *g* for 5 minutes.
6. Remove supernatant and discard.
7. Resuspend the cell pellet containing neurospheres according to the desired method of differentiation:
   - Whole neurospheres plated at low density (e.g. for 3D confocal microscopy on individual intact neurospheres). **For this method, proceed to section 7.5.**
   - Dissociated cells plated at high density (e.g. for 2D qualitative and/or quantitative analyses of total differentiated cells). **For this method, continue to step 8.**
8. Resuspend neurospheres in 200 µL Complete NeuroCult™ Differentiation Medium.
9. Using a 200 µL pipettor set at ~180 µL, triturate neurospheres by pipetting up and down with a pipettor until a single-cell suspension is achieved (approximately 20 - 30 times). Triturate vigorously, but do not introduce air bubbles into the cell suspension.
10. If undissociated neurospheres remain, allow to settle for 1 - 2 minutes and pipette off supernatant containing single cells into a new tube. Repeat trituration by adding 200 µL Complete NeuroCult™ Differentiation Medium to the remaining undissociated neurospheres and continue to triturate as described in step 9. Once a single-cell suspension has been obtained, pool all cell suspensions.
11. Centrifuge at 150 x *g* for 5 minutes.
12. Remove supernatant and resuspend the cells by brief trituration in an appropriate volume (e.g. 1 mL) of Complete NeuroCult™ Differentiation Medium.
13. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer. Proceed to section 7.3 for plating cells.

7.2 Harvesting Cells from Adherent Monolayer Cultures for Differentiation

1. Harvest cells when the culture is 60 - 80% confluent. Use a pipette to remove the medium from the culture vessel.
2. Add PBS to wash the cells as follows:
   - One well of a 6-well plate: 3 mL PBS
   - T-25 cm^2 flask: 10 mL PBS
3. Swirl the culture plate or flask gently, then remove PBS and discard.
4. Add ACCUTASE™ as follows:
   - One well of a 6-well plate: 0.5 mL ACCUTASE™
   - T-25 cm^2 flask: 1 mL ACCUTASE™
5. Incubate at room temperature (15 - 25°C) or at 37°C for 5 minutes.
6. Observe the culture to determine if the cells are starting to detach and detachment is complete. If cells have not completely detached after 5 minutes, dispense a stream of medium across the surface of the cultureware to detach the cells.
7. Using a disposable pipette, add Complete NeuroCult™ Differentiation Medium to the detached cells as follows:
   - One well of a 6-well plate: 2 mL medium
   - T-25 cm^2 flask: 5 mL medium

   Note: This is a wash step to remove the cytokines.
8. Using the same pipette, resuspend the detached cells by pipetting the cell/medium suspension up and down 2 - 3 times.
9. Collect the cells and place in a new sterile 15 mL conical tube. If cells remain in the vessel, add a small volume of Complete NeuroCult™ Differentiation Medium and repeat the procedure to collect the remaining cells.
10. Centrifuge at 110 x *g* for 5 minutes.
11. Remove the supernatant. Using a plastic disposable pipette tip attached to a 200 µL pipettor set at ~180 µL, resuspend cells in a maximum of 200 µL Complete NeuroCult™ Differentiation Medium, pipetting up and down until a single-cell suspension is achieved.
12. Resuspend cells in an appropriate volume (approximately 0.5 - 1 mL) of Complete NeuroCult™ Differentiation Medium.
13. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.

7.3 Plating Cells for Differentiation

1. Plate single-cell suspensions of **embryonic** mouse CNS-derived cells in Complete NeuroCult™ Differentiation Medium as indicated in Table 13.

Table 13. Plating Conditions for Differentiation Cultures of EMBRYONIC Mouse CNS-Derived Cells

CULTUREWARE	SUBSTRATE	VOLUME OF MEDIUM	TOTAL # OF CELLS
24-well plate with coated glass coverslips	PDL/Laminin or PLO/Laminin	1 mL	**Primary cells:** 5 x 10^5 viable cells/well **Subsequent passages:** 1.25 x 10^5 cells/well
Corning® BioCoat™ 8-Well Culture Slide	PDL/Laminin or PDL	0.8 mL	**Primary cells:** 3.2 x 10^5 viable cells/well **Subsequent passages:** 8 x 10^4 viable cells/well

OR

Plate single-cell suspensions of **adult** mouse CNS-derived cells in Complete NeuroCult™ Differentiation Medium into individual wells of a 24-well plate containing Matrigel®-coated coverslips as indicated in Table 14.

Table 14. Plating Conditions for Differentiation Cultures of ADULT Mouse CNS-Derived Cells

CULTUREWARE	SUBSTRATE	VOLUME OF MEDIUM	TOTAL # OF CELLS
24-well plate with coated glass coverslips	Corning® Matrigel®	1 mL	**Primary cells:** 5 x 10^5 viable cells/well **Subsequent passages:** 1 x 10^5 viable cells/well

2. Incubate at 37°C and 5% CO_2 in a humidified incubator.
3. Check cultures daily to determine if the medium needs to be changed during the differentiation procedure. If the medium becomes acidic (turns yellow), perform a half-medium change by removing approximately half of the medium and replacing with fresh Complete NeuroCult™ Differentiation Medium.
4. Observe cultures 6 - 8 days after plating using an inverted light microscope to determine if cells have differentiated. When differentiated, single cells will have spread out and heterogeneous cell morphologies will be observed.
5. Coverslips or coated culture slides containing differentiated neural cells can be removed and processed for indirect immunofluorescence as described in section 15.0.

7.4 Alternate Differentiation Method (bFGF-Stimulation)

To stimulate the proliferation of neuronal progenitor cells prior to differentiation, bFGF can be added to culture medium during the initial stage of the differentiation procedure. This method has been optimized for **adult** mouse CNS-derived cells.

1. Perform steps 1 - 13 in section 7.1 (for neurosphere cultures) or section 7.2 (for adherent monolayer cultures) as described, but in all cases where Complete NeuroCult™ Differentiation Medium is used, replace with NeuroCult™ Proliferation Medium without cytokines (refer to section 3.1.3 steps 1 - 3).

2. To prepare the bFGF-containing medium, add bFGF to NeuroCult™ Proliferation Medium without cytokines to achieve a final concentration of 20 ng/mL.
3. Dilute the cells into the bFGF-containing medium to 1 - 5 x 10^5 viable cells/mL. Add 1 mL per well of a 24-well plate containing Matrigel®-coated glass coverslips.
4. Incubate at 37°C and 5% CO_2 in a humidified incubator overnight.

   Note: The cells are initially cultured in serum-free conditions in the presence of bFGF to promote proliferation of some neuronal progenitor cells.
5. The next day (Day 2), remove the culture medium.
6. Carefully add 1 mL of Complete NeuroCult™ Differentiation Medium to each well.

   Note: The cells are then cultured in serum-containing conditions in the absence of mitogens to limit proliferation and induce differentiation.
7. Incubate at 37°C and 5% CO_2 in a humidified incubator.
8. Check cultures daily to determine if the medium needs to be changed during the differentiation procedure. If the medium becomes acidic (turns yellow), perform a half-medium change by removing approximately half of the medium and replacing with fresh Complete NeuroCult™ Differentiation Medium.
9. Observe cultures 6 - 14 days after plating using an inverted light microscope to determine if cells have differentiated.
10. Coverslips containing differentiated neural cells can be removed and processed for indirect immunofluorescence as described in section 15.0.

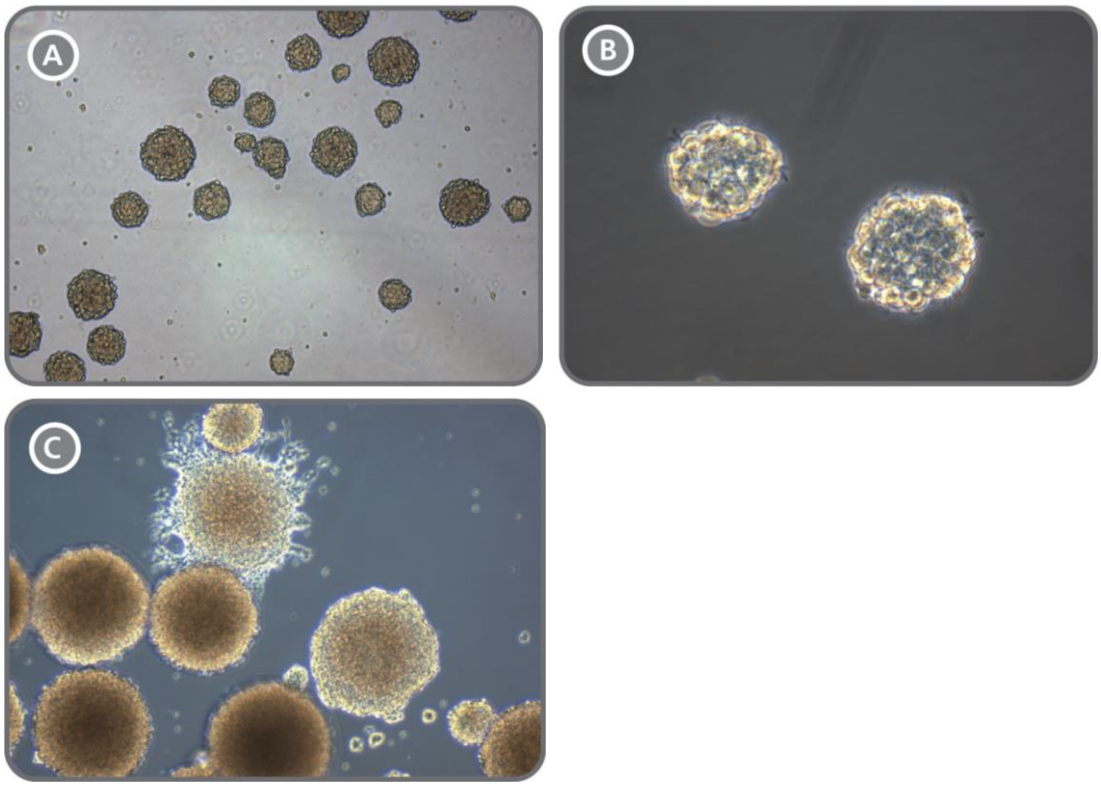
7.5 Differentiation of Cells Present in Intact Neurospheres

1. Continuing from section 7.1 step 7, resuspend neurospheres (do not triturate) in 5 mL Complete NeuroCult™ Differentiation Medium.
2. Transfer the neurospheres into a 60 mm dish (or another appropriate vessel) to allow isolation of individual neurospheres with a disposable plastic pipette tip.
3. Dispense 1 mL of Complete NeuroCult™ Differentiation Medium into individual wells of a 24-well plate containing glass coverslips coated with either PLO/laminin or PDL/laminin.
4. Isolate 1 - 10 neurospheres with a pipette and deposit into the individual wells of a 24-well plate containing glass coverslips coated with either PLO/laminin or PDL/laminin.

   Note: While individual neurospheres are generally visible to the naked eye, this procedure can also be performed using an appropriate microscope placed in the biohazard safety cabinet.
5. Incubate at 37°C and 5% CO_2 in a humidified incubator.
6. Check cultures daily to determine if the medium needs to be changed during the differentiation procedure. If the medium becomes acidic (turns yellow), perform a half-medium change by removing approximately half of the medium and replacing with fresh Complete NeuroCult™ Differentiation Medium.
7. Observe cultures 6 - 8 days after plating using an inverted light microscope to determine if cells have differentiated. When differentiated, intact neurospheres will have spread out and heterogeneous cell morphologies will be observed.
8. Coverslips or coated culture slides containing differentiated neural cells can be removed and processed for indirect immunofluorescence as described in section 15.0.

8.0 Mouse: Representative Images of Neural Stem and Progenitor Cell Cultures

8.1 Mouse Neurosphere Cultures



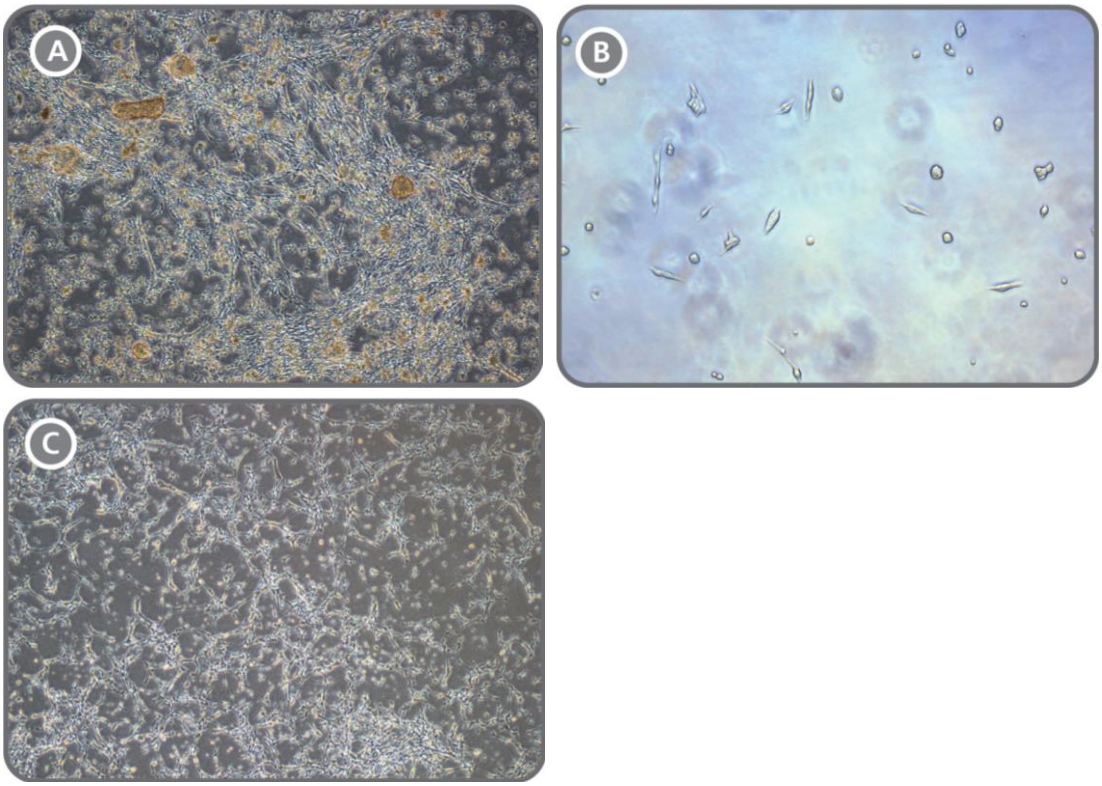
A - C. "Good-Quality" and "Bad-Quality" Neurosphere Cultures

A. Day 5 Adult Mouse CNS-Derived Cells. Neurospheres have good morphology, showing round periphery and phase contrast bright cells.
Magnification: 10X

B. Day 5 Adult Mouse CNS-Derived Cells. Neurospheres have good morphology, showing round periphery, presence of microspikes, and phase contrast bright cells.
Magnification: 20X

C. Day 10 Adult Mouse CNS-Derived Cells. Neurospheres have bad morphology, showing dark center cores and some cell attachment to the tissue culture flasks. These cells were incubated beyond the optimal culture period of 5 - 7 days.
Magnification: 10X

8.2 Mouse Adherent Monolayer Cultures



A - C. Adherent Monolayer Cultures of Adult Mouse Subventricular Zone (SVZ) Cells

A. Day 7 Culture of Primary Adult Mouse SVZ-Derived Cells
Note: In primary cultures, debris can sometimes make it difficult to see the adherent cells.
Magnification: 10X

B. Day 2 of Passage 2 Adult Mouse SVZ-Derived Cell Cultures
Adherent cells are present, however some cellular aggregates are still observed in suspension. In comparison to the primary cultures, cultures that have undergone passaging are free of cell debris.
Magnification: 20X

C. Day 7 of Passage 2 Adult Mouse SVZ-Derived Cell Cultures
Adherent cells have formed a monolayer of cells that is approximately 70 - 80% confluent and ready for passaging.
Magnification: 10X

9.0 Rat: Required Materials and Equipment

9.1 Dissociation of Rat CNS Tissue

- Phosphate-buffered saline (PBS) containing 2% glucose (required for collecting tissue)
- Trypan Blue (Catalog #07050)
- NeuroCult™ Enzymatic Dissociation Kit for Adult CNS Tissue (Mouse & Rat; Catalog #05715) [optional]

9.2 Proliferation of Rat Neural Stem and Progenitor Cells

- Human Recombinant EGF (100 µg, Catalog #78006.1)
- Human Recombinant bFGF (10 µg, Catalog #78003.1)
- Heparin Solution (Catalog #07980)
- Bovine serum albumin (BSA) or human serum albumin (HSA) (required for reconstitution of EGF and bFGF)
- Trypan Blue (Catalog #07050)
- T-25 cm^2 flask:

  Nunc™ EasYFlask™ (Thermo Fisher Catalog #156367 or VWR Catalog #15708-130)

  OR

  Corning® Cell Culture Flask (Corning Catalog #430639 or VWR Catalog #29186-010)

9.3 Differentiation of Rat Neural Stem and Progenitor Cells

- Adhesive substrates recommended for differentiation of rat central nervous system (CNS) cells:
- Poly-L-Ornithine (PLO) Solution (Sigma Catalog #P4957)
- Poly-D-Lysine (PDL; Sigma Catalog #P7280)
- Laminin (Sigma Catalog #L2020)

Table 15: Recommended Cultureware for Differentiation

CULTUREWARE	RECOMMENDED SUPPLIERS
8-well culture slide	Corning® BioCoat™ Poly-D-Lysine 8-Well Culture Slides (Corning Catalog #354632) Corning® BioCoat™ Poly-D-Lysine/Laminin 8-Well Culture Slides (Corning Catalog #354688)
Coverslip in a 24-well culture dish	12 mm round glass coverslip (Carolina Biological Catalog #633029) Costar® 24-Well Flat-Bottom Plate, Tissue Culture-Treated (STEMCELL Catalog #38017)

9.4 Immunolabeling Differentiated Cells

- D-PBS (Without Ca++ or Mg++) (PBS; Catalog #37350)
- 4% Paraformaldehyde (in PBS pH 7.2; Sigma Catalog #P6148)
- Triton™ X-100 (Sigma Catalog #T-9284)
- Mounting medium (e.g. FluorSave™ Reagent; EMD Millipore Catalog #345789)

9.5 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Benchtop centrifuge
- Vortex
- Forceps
- Pipette-aid
- Pipettors: 10 µL (Catalog #38061), 200 µL (Catalog #38059), and 1 mL (Catalog #38058) with sterile disposable plastic pipette tips
- Hemocytometer
- Routine light microscope for hemocytometer cell counts
- Inverted microscope with total magnification of approximately 20 - 30X, 80X, and 125X
- 24-well tissue culture plate (e.g. Catalog #38017)
- 15 mL polypropylene conical tubes (Catalog #38009)

10.0 Rat: Preparation of Reagents and Cultureware

10.1 For Proliferation of Neural Stem and Progenitor Cells

10.1.1 EGF Stock Solution (10 µg/mL)

1. Add 1 mL of sterile water containing at least 0.1% BSA to EGF (100 µg/vial) by pipetting the solution down the sides of the vial. Do not vortex.

   Note: If not used immediately, store 100 µg/mL EGF at -20°C to -80°C for up to 6 months.

2. Dilute EGF (100 µg/mL) 1 in 10 with sterile water containing at least 0.1% BSA (final concentration 10 µg/mL).

   Note: If not used immediately, aliquot (0.1 - 0.3 mL) and store at -20°C. Do not freeze/thaw each aliquot more than 3 times.

10.1.2 bFGF Stock Solution (10 µg/mL)

1. Add 1 mL of sterile water containing at least 0.1% BSA to bFGF (10 µg/vial) by pipetting the solution down the sides of the vial. Do not vortex.

   Note: If not used immediately, aliquot (0.1 - 0.3 mL) and store at -20°C. Do not freeze/thaw each aliquot more than 3 times.

10.1.3 Complete NeuroCult™ Proliferation Medium

1. Thaw one 50 mL bottle of NeuroCult™ Proliferation Supplement at 2 - 8°C overnight or at 37°C for 1 - 2 hours.

   Note: If not used immediately, aliquot into 10 mL volumes and store at -20°C. After thawing aliquots, use immediately. Do not re-freeze. A white precipitate may form after storage at -20°C. The precipitate will disappear after complete thawing at 37°C and mixing the contents.

   Note: If the supplement thawed during shipping, immediately aliquot and store at -20°C. If the supplement remained cool (below ~10°C), performance will be unaffected.

2. Add the entire volume (50 mL) of NeuroCult™ Proliferation Supplement to 1 bottle (450 mL) of NeuroCult™ Basal Medium.

   OR

   Add 1 mL NeuroCult™ Proliferation Supplement to every 9 mL NeuroCult™ Basal Medium.

3. Mix thoroughly. **NeuroCult™ Proliferation Medium <u>without cytokines</u>** is now ready for use.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month. Avoid repeated exposure of medium to room temperature and light.

   Note: NeuroCult™ Proliferation Medium without cytokines is only used in very specific steps in the procedure, e.g. when resuspending and washing cell suspensions. Do not use this cytokine-free medium for cell culture.

4. To each 10 mL of NeuroCult™ Proliferation Medium without cytokines (from step 3), add the following:
   - 20 µL of 10 µg/mL EGF (to give a final concentration of 20 ng/mL EGF)
   - 10 µL of 10 µg/mL bFGF (to give a final concentration of 10 ng/mL bFGF)
   - 10 µL of Heparin Solution (to give a final concentration of 0.0002% Heparin (w/v); equals 2 µg/mL)

   This will be referred to as **Complete NeuroCult™ Proliferation Medium**.

   Note: If not used immediately, store Complete NeuroCult™ Proliferation Medium at 2 - 8°C for up to 2 weeks.

10.2 For Differentiation of Neural Stem and Progenitor Cells

10.2.1 Complete NeuroCult™ Differentiation Medium

1. Thaw one 50 mL bottle of NeuroCult™ Differentiation Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Note: If not used immediately, aliquot into 10 mL volumes and store at -20°C. Once aliquots are thawed, use immediately. Do not re-freeze.

2. Add the entire volume (50 mL) of NeuroCult™ Differentiation Supplement to 1 bottle (450 mL) of NeuroCult™ Basal Medium.

   OR

   Add 1 mL NeuroCult™ Differentiation Supplement to every 9 mL NeuroCult™ Basal Medium (1 in 10 dilution).

3. Mix thoroughly. Complete NeuroCult™ Differentiation Medium is now ready for use.

   Note: If not used immediately, store Complete NeuroCult™ Differentiation Medium at 2 - 8°C for up to 1 month.

10.2.2 Coating Coverslips for Differentiation Cultures

If using round glass coverslips for immunocytochemistry, ensure that the chosen coverslips fit easily in and out of the wells of the plate used for culturing cells. Sterilize coverslips by autoclaving prior to coating.

Coat coverslips with either poly-L-ornithine (PLO)/laminin or poly-D-lysine (PDL)/laminin, as described below.

1. Preparation of PLO or PDL stock solutions

Preparation of 15 µg/mL PLO Stock Solution

Add 1.5 mL Poly-L-Ornithine Solution (0.01%) to 8.5 mL sterile PBS.

OR

Preparation of 100 µg/mL PDL Stock Solution

a. Dissolve 5 mg PDL in 50 mL sterile water.
b. Aliquot solution into polypropylene vials and store at 2 - 8°C.

2. Preparation of 10 µg/mL laminin stock solution

a. Thaw laminin at 2 - 8°C, to prevent laminin from gelling.
b. Prepare a 10 µg/mL working solution of laminin by diluting the laminin in sterile PBS or sterile water (the amount prepared should correspond to the amount needed for immediate use).
c. Store the remaining laminin (which has not been diluted) in appropriately sized aliquots at -20°C.

3. Preparation of coated coverslips

a. Using sterile forceps, transfer one sterile glass coverslip per well of a 24-well plate.

For PLO:

Dispense 0.5 - 1 mL of the 15 µg/mL PLO stock solution into each well containing a coverslip.

For PDL:

Dilute the 100 µg/mL PDL stock solution with sterile water to a final concentration of 10 µg/mL. Dispense 0.5 - 1 mL of the 10 µg/mL PDL solution into each well containing a coverslip.

Note: Ensure that the coverslips are completely submerged in the PLO or PDL solution. If necessary, use a plastic disposable pipette tip to push the coverslip to the bottom of the well.

b. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight (~20 hours).

c. Wash each well with 2 x 1 mL sterile PBS. Remove as much of the PBS as possible.

d. Dispense 0.5 - 1 mL of the 10 µg/mL laminin stock solution into each well containing a coverslip.

e. Incubate at room temperature (15 - 25°C) for 2 hours or at 2 - 8°C overnight.

f. Remove the solution from each well. Wash each well with 3 x 1 mL sterile PBS (15 minutes for each wash) to remove any residual laminin solution.

Note: Only remove PBS when ready to plate the cells. Do not let the coated plates dry completely.

4. The substrate-coated coverslips are ready for use. If not used immediately, wrap the plate with Parafilm®, do not remove PBS from the wells, and store the plate at 2 - 8ºC for up to 2 weeks.

11.0 Rat: Preparation of E18 CNS Cells and Initial Culture of Neurospheres

1. Dissect rat embryos (e.g. Sprague/Dawley or Fischer 344) at gestational day E18, where day E0 is the day a gestational plug forms.
2. Remove the entire brain from the embryos and transfer to a 35 mm dish containing **cold** PBS containing 2% glucose [Refer to Cell Biology: A Laboratory Handbook. ed. Julio E. Cells. 1998. Volume 1, p149 for additional information].
3. Dissect out desired brain regions and place in **cold** PBS containing 2% glucose on ice.
4. When dissections are complete, transfer all tissue in PBS containing 2% glucose into a 15 mL conical tube or 14 mL round-bottom tube.
5. Allow tissue to settle and pipette off supernatant. Resuspend tissue in 1 mL Complete NeuroCult™ Proliferation Medium (prepared in section 10.1.3).
6. Using a disposable plastic tip attached to a 1 mL pipettor set at 0.9 mL, triturate the tissue approximately 5 times. If undissociated tissue remains, allow the clumps to settle for 1 - 2 minutes. Pipette off the supernatant containing the single cells into a new 15 mL conical tube. Discard undissociated tissue.

   Note: Triturate to create a single-cell suspension, but be careful to not create air bubbles. The majority of the tissue will be dissociated in this step. Do not excessively triturate, as this will result in increased cell death.
7. Add 10 mL of Complete NeuroCult™ Proliferation Medium to the single-cell suspension.
8. Centrifuge at 150 x *g* for 5 minutes. Remove and discard supernatant.
9. Resuspend cells with a brief trituration (2 times) with a disposable plastic pipette tip in 1 mL Complete NeuroCult™ Proliferation Medium. Add an additional 2 mL of Complete NeuroCult™ Proliferation Medium.
10. Filter the cell suspension through a 37 µm strainer (e.g. Catalog #27215).
11. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.
12. Seed cells at a density of 6 x 10^4 viable cells/mL or 1.2 x 10^5 viable cells/mL (see note in section 12.3) in a T-25 cm^2 tissue culture flask. Use approximately 10 mL of Complete NeuroCult™ Proliferation Medium.
13. Incubate at 37°C and 5% CO_2. Refer to section 14.1 for images of rat neurospheres 1 and 2 days after plating.

Note: A partial medium change (25 - 30% of total volume) is highly recommended 2 - 3 days after plating, to prevent the medium from becoming acidic. To change the medium, position the flask in an upright position and let the cells and spheres settle to the bottom (2 - 3 minutes). Slowly remove ~3 mL of medium, being careful not to remove the cells, and replace with 3 mL of fresh Complete NeuroCult™ Proliferation Medium.

12.0 Rat: Subculture of Neurospheres

Cultures of rat E18 cortical or striatal neurospheres should be passaged every 3 - 4 days. Neurospheres should be passaged before the diameter exceeds 100 µm to avoid hypoxic cells in the centre of the spheres. Refer to section 14.1 Figure 3 for a photo of neurospheres ready to be passaged. Cells will proliferate as spheroids, called neurospheres, which usually detach from the surface of the flask and float in suspension. The neurospheres should be ready for subculture 3 - 4 days after plating, depending on sphere density and size. Viable neurospheres will, for the most part, be semi-transparent and phase contrast bright, with many of the cells on the outer surface displaying microspikes.

1. Pre-wet a 10 mL disposable pipette with Complete NeuroCult™ Proliferation Medium. Remove neurospheres and medium and place in an appropriately sized sterile tissue culture tube (e.g. 15 mL conical tube).
2. Centrifuge at 90 x *g* for 5 minutes.
3. Dissociate neurospheres using either mechanical dissociation (section 12.1) or ACCUTASE™ enzymatic dissociation (section 12.2).

12.1 Mechanical Dissociation

1. Remove supernatant, leaving behind approximately 150 - 180 µL of medium. Set the volume of a 200 µL pipettor to slightly less than the approximate volume of the remaining medium (e.g. if the volume of remaining medium is 180 µL, set the volume of the pipettor to 160 µL). Pre-wet the tip with medium to reduce cells sticking to the walls of the tip.
2. Gently triturate the cell pellet 10 - 15 times. Slightly tilt the pipette tip and press it against the bottom or side of the tube to generate resistance in order to break up the neurospheres. Rinse the side of the tube during trituration to remove the remaining neurospheres that are attached to the side of the tube. If some neurospheres remain undissociated after 15 triturations (this usually occurs at later passages) trituration can be extended to a maximum of 25 - 35 times. To maintain high cell viability, avoid using a fire-polished glass pipette to disaggregate the neurospheres.
3. Measure the volume of the cell suspension. Count viable cells using Trypan Blue (1 in 10 dilution at earlier passages, lower dilution for later passages) and a hemocytometer.

   Note: To avoid passaging cells as clumps, pipette cells up and down with a 200 µL pipette tip 4 - 8 times to obtain a single-cell suspension prior to seeding cells in a flask.
4. Proceed to section 12.3 for replating cells.

12.2 ACCUTASE™ Enzymatic Dissociation

ACCUTASE™ contains both proteolytic and collagenolytic enzymes and is useful for the routine dissociation of cells.

1. Thaw ACCUTASE™ at 2 - 8 °C overnight, in a vessel of cool water, or at room temperature (15 - 25°C) only until thawed. Do not thaw at 37°C.
2. Following centrifugation of neurospheres (section 12.0 step 2), remove and discard the supernatant, leaving behind the cell pellet.
3. Pre-wet the disposable pipette tip with Complete NeuroCult™ Proliferation Medium before dissociating cells, to prevent the cells from sticking to the walls of the tip.
4. Add 200 µL of ACCUTASE™ to each cell pellet harvested from a T-25 cm^2 flask.
5. Incubate at room temperature (15 - 25°C) or at 37°C for 5 minutes. At the mid-point of the incubation, mix by gently shaking the tube to ensure the cell suspension is well mixed.
6. After 5 minutes, determine by eye if there are any undissociated neurospheres or aggregates remaining. If clumps remain, perform a gentle trituration step using a 200 µL pipettor set to 180 µL.

7. Wash cells with Complete NeuroCult™ Proliferation Medium.
8. Centrifuge at 150 x *g* for 5 minutes. Discard the supernatant.
9. Resuspend cells in ~0.5 mL of Complete NeuroCult™ Proliferation Medium.
10. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer. Proceed to section 12.3 for replating cells.

12.3 Replating Cells for Neurosphere Cultures

1. Seed cells at a density of 1.25 x 10^4 or 2.5 x 10^4 viable cells/mL (see note below) in a T-25 cm^2 tissue culture flask containing 10 mL Complete NeuroCult™ Proliferation Medium.
2. Incubate at 37°C and 5% CO_2.
3. Examine cultures under the microscope regularly. Perform a partial medium change 2 days after the cultures are set up (see note in section 11.0).

Note: Neural stem and progenitor cells from rat CNS tissue have different growth characteristics compared to mouse neural stem and progenitor cells. The rate of proliferation of E18 rat cortical is higher (varies from 2 to > 20-fold expansion of total cells every 3 - 4 days of culture) during early passages (P2 - P5). A critical phase in the growth curve occurs between P5 and P9 (20 - 36 days) in culture, when a significant decrease in fold expansion (average 2- to 5-fold) is observed. During this critical period, cells start to attach to the flask and differentiate. To reduce attachment of spheres to the flask, it is important to perform partial medium changes every 2 days, being careful not to disrupt the attached cells so that only the floating spheres are passaged. Culture more than one flask during the critical passages (P5 - P9); use two different seeding densities (1.25 x 10^4 cells/mL and 2.5 x10^4 cells/mL) to ensure a sufficient number of cells are obtained for the next passage.

13.0 Rat: Differentiation of Neural Stem and Progenitor Cells

1. If using the Corning® BioCoat™ 8-well culture slide (coated with poly-D-lysine/laminin or poly-D-lysine) add 0.75 mL/well of Complete NeuroCult™ Differentiation Medium.

   OR

   If using PDL/laminin- or PLO/laminin-coated glass coverslips prepared as described in section 10.2.2, place a single coated coverslip into an individual well of a 24-well culture dish containing 1 mL of Complete NeuroCult™ Differentiation Medium.
2. After 3 - 4 days of culturing rat neurospheres in Complete NeuroCult™ Proliferation Medium, remove the medium with suspended cells and place in an appropriately sized sterile tissue culture tube. Centrifuge at 90 x *g* for 5 minutes.
3. Remove the supernatant and discard.
4. Resuspend the neurospheres in 150 - 180 µL of Complete NeuroCult™ Differentiation Medium. Using a pipettor with a 200 µL pipette tip, triturate the neurosphere suspension until a single-cell suspension is achieved (refer to section 12.1 for a description of trituration).
5. Resuspend the single-cell suspension in 10 mL Complete NeuroCult™ Differentiation Medium. Centrifuge at 150 x *g* for 5 minutes. Remove the supernatant and discard.
6. Resuspend the cell pellet in approximately 200 µL of Complete NeuroCult™ Differentiation Medium.
7. Measure the volume and count cells using Trypan Blue (1 in 5 or 1 in 10 dilution) and a hemocytometer.
8. Prepare cells in an appropriate volume of Complete NeuroCult™ Differentiation Medium. Plate cells with a density of 5 x 10^5 cells/mL on a coated coverslip in a 24-well plate or at 1 x 10^5 cells/well in a BioCoat™ 8-well chamber slide.
9. Observe cultures after 5 - 10 days with an inverted light microscope and determine if cells have differentiated and are viable.
10. Check cultures routinely to determine if the medium needs to be changed during the differentiation procedure. If the medium becomes acidic (yellow), perform a half-medium change by removing approximately 50% of the medium and replacing with fresh Complete NeuroCult™ Differentiation Medium.
11. Coverslips or coated chamber slides containing differentiated rat neural cells can be removed after 5 - 10 days and processed for indirect immunofluorescence as described in section 15.0.

14.0 Rat: Representative Images of Neural Stem and Progenitor Cell Cultures

14.1 Rat Neurospheres

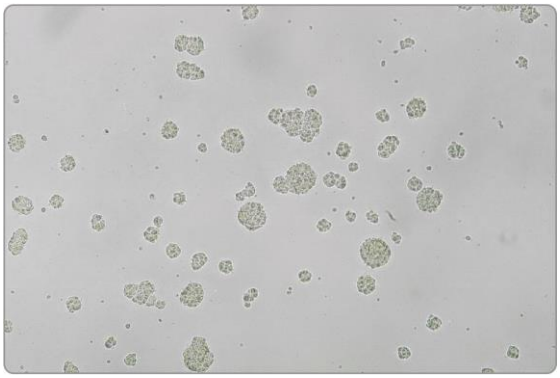


Figure 1. Small neurospheres, one day after plating single-cell suspensions of rat E18 cortex in Complete NeuroCult™ Proliferation Medium.
Magnification: 125X

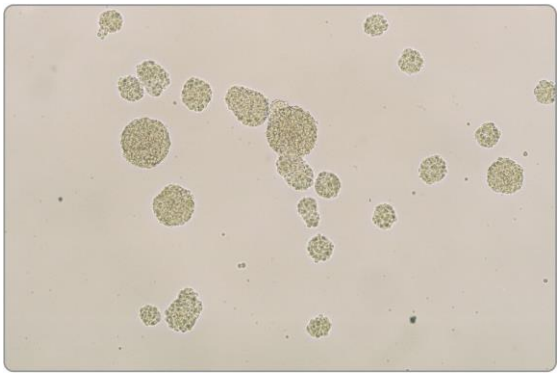


Figure 2. Neurospheres derived from rat E18 cortical cells after 2 days of culture in Complete NeuroCult™ Proliferation Medium. The size (diameter) of the neurospheres has increased.
Magnification: 125X

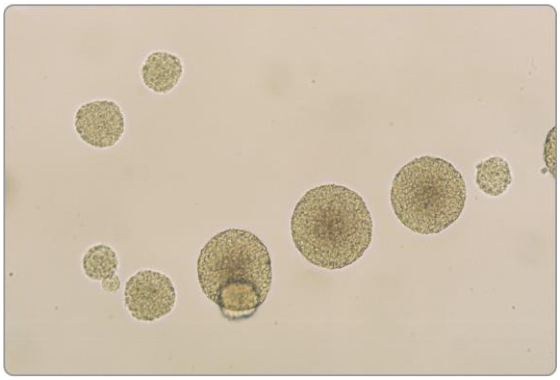


Figure 3. Neurospheres derived from rat E18 cortical cells after 4 days in culture with Complete NeuroCult™ Proliferation Medium. Neurospheres are ready to be passaged. If not passaged, cells in the center of the spheres will become dark and hypoxic and the spheres will stick down to the bottom of flask.
Magnification: 125X

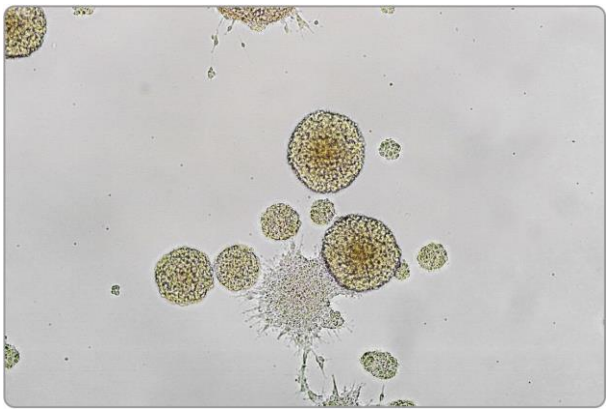


Figure 4. The critical phase in rat neurosphere cultures: Passage 5 - 9 (20 - 36 days in culture). At this point, some neurospheres will adhere to the flask and start differentiating. It is important to perform a partial medium change every other day and passage only the floating neurospheres (to prevent differentiation).
Magnification: 125X

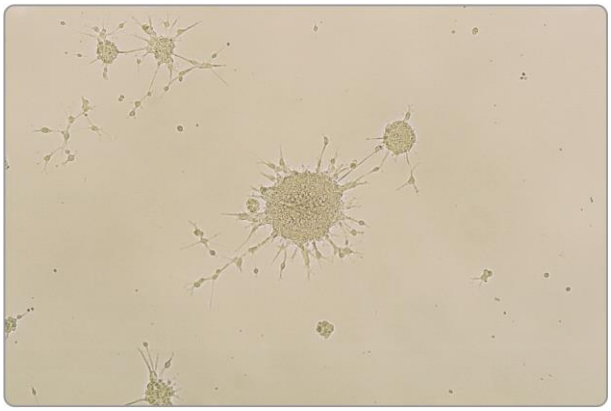


Figure 5. Neurospheres derived from rat E18 cortical cells which have all adhered and differentiated after 3 days in culture. The medium should be regularly replenished and only floating neurospheres passaged to prevent cells attaching to the flask.
Magnification: 125X

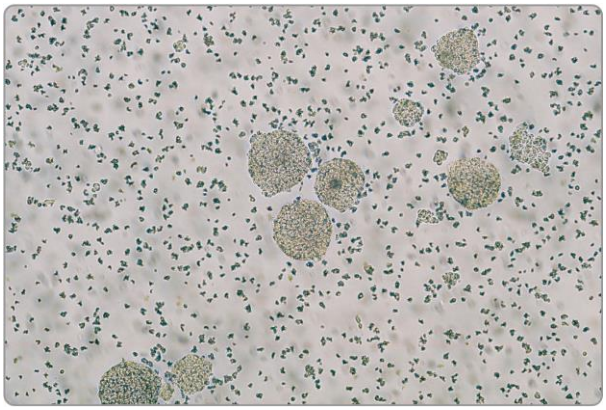


Figure 6. “Unhealthy” neurospheres derived from rat E18 cortical cells after 4 days in culture. Individual neurospheres are beginning to dissociate into single cells in culture, an indication of a problem with the culture conditions or cells.
Magnification: 125X

14.2 Immunofluorescent Labeling to Identify Differentiated Cells Obtained from Rat Neurospheres

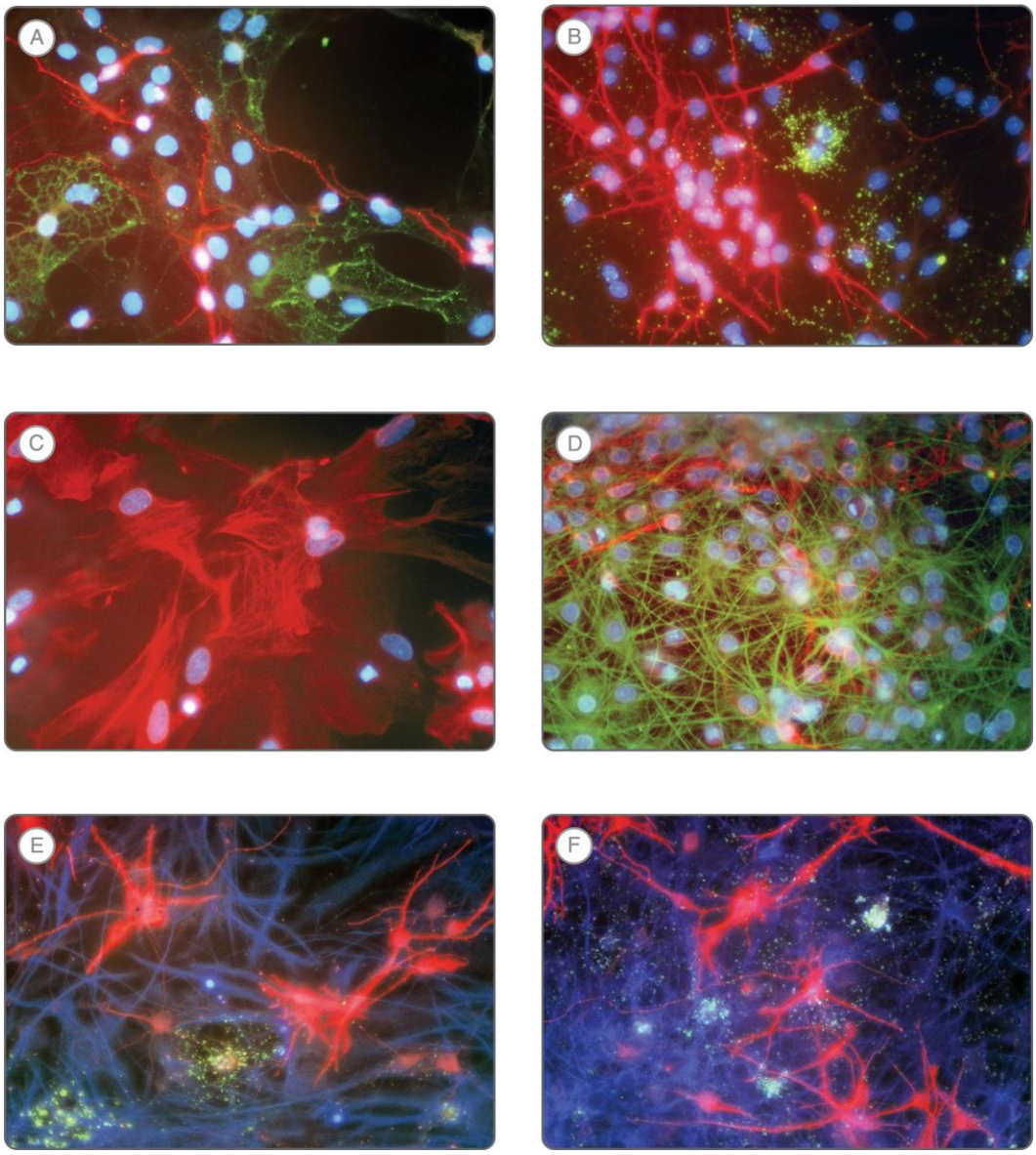


Figure 7. Differentiated Rat Neural Cells Cultured with NeuroCult™ Differentiation Medium
Cells are immunolabeled with lineage-specific antibodies as described in section 15.0 and counterstained with DAPI (blue).
Magnification: 400X
A. β-Tubulin (red) and MBP (Green)
B. Oligodendrocyte Marker O4 (green) and β-Tubulin (red)
C. Nestin (red)
D. GFAP (green) and MAP2 (red)
E & F. Triple labeling with Oligodendrocyte Marker O4 (green), GFAP (blue) and β-Tubulin (red)

15.0 Immunolabeling to Identify Differentiated Cell Types

15.1 Fixation

1. Remove approximately 90% of the culture medium from each well.

   Note: Do not remove all of the culture medium prior to fixation; the unfixed cells should not be exposed to air.

2. Working in a chemical fume hood, add the following volume of 4% paraformaldehyde (in PBS, pH 7.2) to each well:
   - 8-well culture slide: 0.5 mL
   - 24-well plate: 1 mL
3. Incubate at room temperature (15 - 25°C) for 30 minutes.
4. Working in a chemical fume hood, remove the paraformaldehyde solution.
5. Add 1 mL PBS (pH 7.2) to each well and incubate at room temperature (15 - 25°C) for 5 minutes. Remove PBS.
6. Repeat the PBS wash procedure 2 more times for a total of 3 washes.

15.2 Permeabilization

1. Add the following volume of 0.3% Triton™ X-100 (in PBS) to each well:
   - 8-well culture slide: 0.5 mL
   - 24-well plate: 1 mL
2. Incubate at room temperature (15 - 25°C) for 5 minutes.
3. Remove Triton™ X-100/PBS.
4. Add 1 mL PBS (pH 7.2) to each well and incubate at room temperature (15 - 25°C) for 5 minutes. Remove PBS.
5. Repeat the PBS wash procedure 2 more times for a total of 3 washes.

15.3 Blocking and Labeling with Primary Antibodies

1. Prepare the blocking solution of 10% serum in PBS. The type of serum used should correspond to the animal in which the secondary antibody was generated.
2. Dilute the primary antibody in blocking solution (containing the appropriate serum) to give an appropriate dilution for immunolabeling. A minimum of 250 µL should be added to each well; this volume is sufficient to coat the entire surface area of coverslips or chamber wells.*

   Note: For a complete list of available antibodies visit www.stemcell.com or contact us at techsupport@stemcell.com.

3. Add diluted primary antibodies to the wells of a 24-well plate or to culture slides.

   Note: If using culture slides, place the culture slide in a hydrating chamber (e.g. a plastic container or box containing a pre-wetted paper towel).

4. Incubate at 37°C for 2 hours or at 2 - 8°C overnight.
5. Wash off primary antibodies with 3 x 5-minute PBS washes. Remove PBS.

*Alternatively, a small volume of antibody (approximately 50 µL) can be added directly on the coverslip containing the differentiated cells and a second clean coverslip can be placed directly on top.

15.4 Secondary Labeling

1. Dilute secondary antibodies in PBS + 2% serum (the same serum used for preparing the blocking solution) to give an appropriate concentration for immunolabeling. Add a minimum volume of 250 µL to each well or slide containing differentiated cells labeled with primary antibodies.
2. Incubate secondary antibodies at 37°C for 30 minutes or at 2 - 8°C overnight.

   Note: Secondary antibodies are sensitive to light; whenever possible, keep samples in the dark to prevent bleaching.
3. Wash off secondary antibody with 3 x 5-minute PBS washes. Remove PBS.
4. After the last wash, add distilled water to each well.

15.5 Mounting

15.5.1 8-Well Culture Slides

1. Follow the manufacturer's protocol for removal of the chambers from the glass slides. Rinse slides in distilled water.
2. Add 5 µL mounting medium (e.g. FluorSave™ Reagent) to each chamber slot and cover with a 75 mm coverslip. Avoid trapping any air bubbles.
3. Visualize immunolabeling under a fluorescent microscope using the appropriate filters for each fluorophore.

15.5.2 Glass Coverslips in 24-Well Plates

1. Add 10 µL mounting medium (e.g. FluorSave™ Reagent) onto a clean glass coverslip. Remove immunolabeled glass coverslip from the 24-well plate and gently tap corner of the coverslip to remove excess water.
2. Place coverslip cell side down onto the mounting medium. Avoid trapping any air bubbles.
3. Visualize immunolabeling under a fluorescent microscope using the appropriate filters for each fluorophore.

TECHNICAL MANUAL

In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™

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