TECHNICAL MANUAL

Chemical Dissociation of Neurospheres Derived From Embryonic and Adult Mouse CNS Tissue Using NeuroCult™ Chemical Dissociation Kit

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1.0 Introduction

Neural stem cells (NSCs) isolated from the embryonic or adult mouse central nervous system (CNS) can be maintained in an undifferentiated proliferative state in vitro by culturing them in a defined serum-free medium supplemented with a mitogen. STEMCELL Technologies has developed the optimized and standardized NeuroCult™ Proliferation Kit (Mouse & Rat; Catalog #05702) for the culture of mouse and rat neural stem and progenitor cells from normal tissues or tumor samples. When cultured in such a medium, NSCs will proliferate in culture to form aggregates of cells or spheres, called neurospheres, which can be dissociated into a single-cell suspension and replated to generate more neurospheres.

Currently the most commonly used method to dissociate neurospheres involves a mechanical dissociation procedure called trituration. Trituration is a rather crude and harsh dissociation method which can result in cell loss and cell death. The manual nature of trituration also makes it difficult to compare measured values (such as percent cell viability) since dissociation efficiency varies for different CNS tissue sources and between individuals. Even in the hands of an experienced researcher, it is not unusual to obtain measured cell viabilities of 60% or less.

STEMCELL Technologies has developed **NeuroCult™ Chemical Dissociation Kit (Mouse)** for the **non-mechanical** and **non-enzymatic dissociation** of neurospheres derived from embryonic or adult mouse CNS tissue (refer to Figure 1 for a procedure overview). Chemical dissociation with this kit results in minimal damage to the cells within neurospheres, allowing for increased viability and an increase in total number of cells generated per passage (compared to trituration; Figure 2), while maintaining the functional properties of the cells upon subsequent subculture (Figure 3).

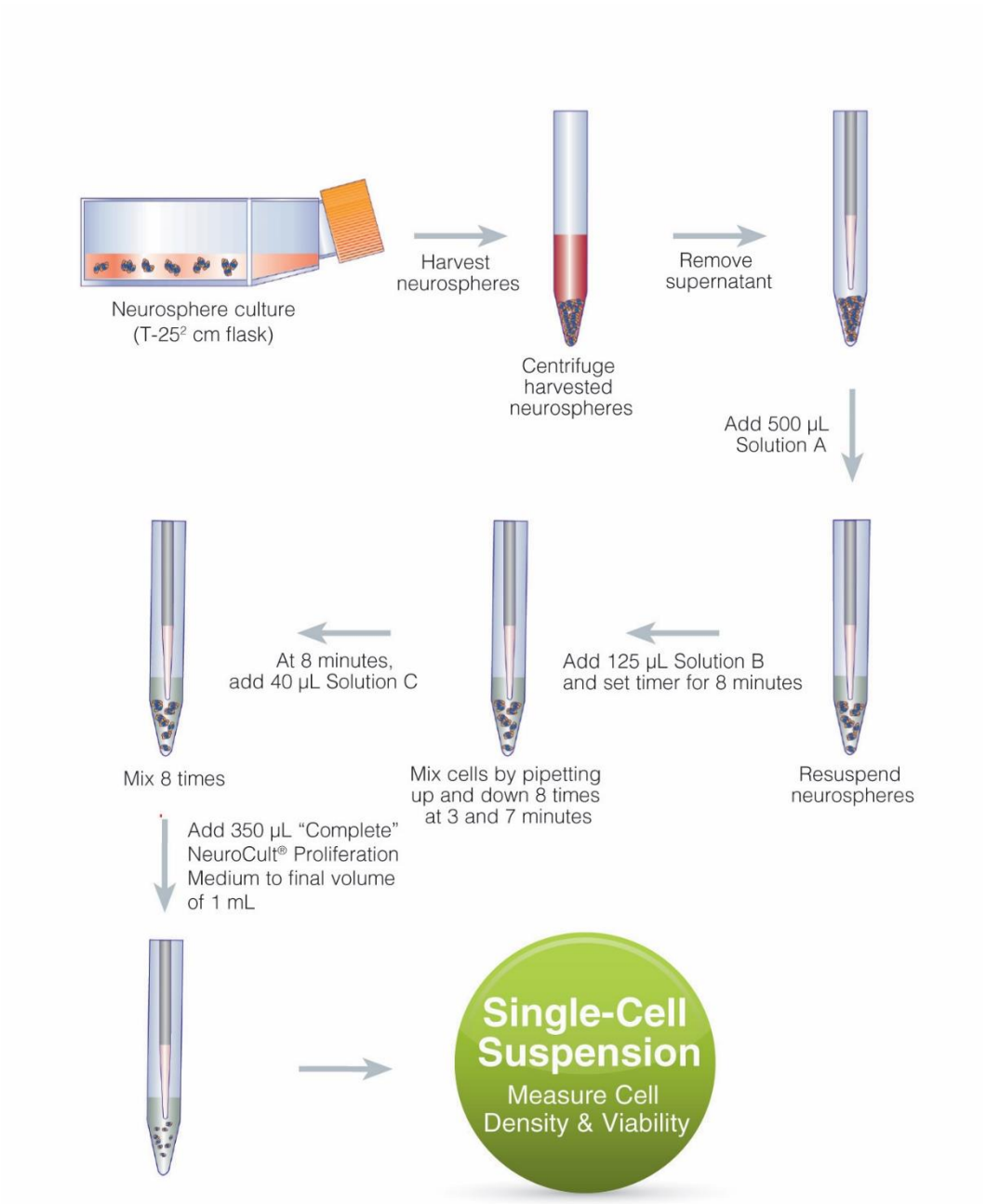

Figure 1. Overview of NeuroCult™ Chemical Dissociation Procedure

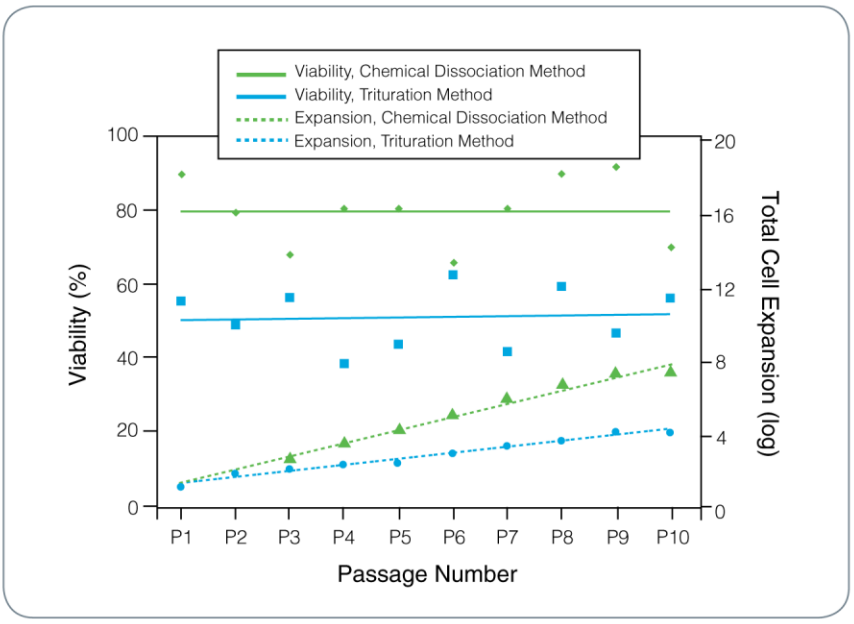

Figure 2. Comparison of Percent Cell Viability and Cell Expansion Between NeuroCult™ Chemical Dissociation Kit and Trituration. Mouse neurospheres were dissociated at each passage (up to P10) with NeuroCult™ Chemical Dissociation Kit or trituration. Cells dissociated with NeuroCult™ Chemical Dissociation Kit had a significantly higher percent viability and total cell number (after expansion) in comparison with trituration (mean; n = 3).

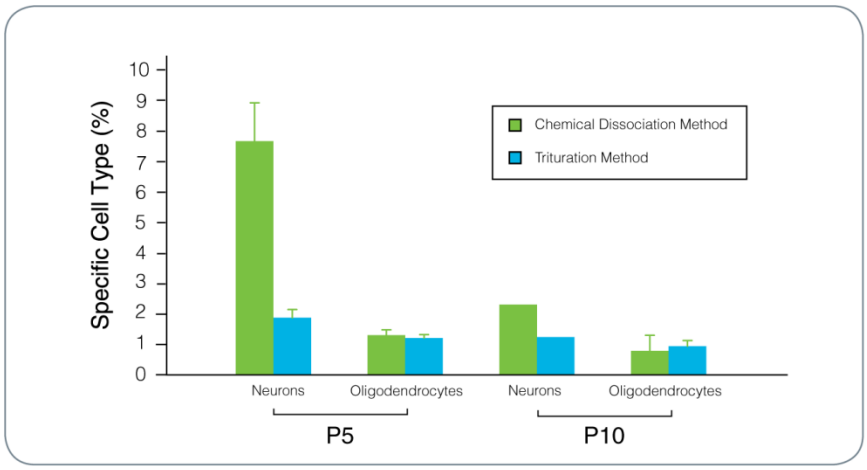

Figure 3. Comparison of the Multi-Lineage Differentiation Potential of Cells Isolated From Neurospheres Dissociated with NeuroCult™ Chemical Dissociation Kit or Trituration
Cell-specific lineages were determined by immunolabeling with specific antibodies for neurons (Anti-Beta-Tubulin III Antibody, Clone TUJ1, Catalog #60052) and oligodendrocytes (anti-myelin basic protein [MBP]) at passages 5 (P5) and 10 (P10). The percentage of astrocytes generated by cells from neurospheres dissociated using either NeuroCult™ Chemical Dissociation Kit or trituration were found to be similar, as determined by immunolabeling with an anti-glial fibrillary acidic protein antibody (Anti-GFAP Antibody, Polyclonal; Catalog #60128 [data not shown]).

2.0 Materials and Equipment Required

2.1 NeuroCult™ Chemical Dissociation Kit (Mouse)

The following are components of NeuroCult™ Chemical Dissociation Kit (Mouse; Catalog #05707) and are not available for individual sale. For storage and stability information, refer to the Product Information Sheet available at www.stemcell.com or contact us to request a copy.

COMPONENT #	COMPONENT NAME	SIZE
05707A	NeuroCult™ Chemical Dissociation Solution A	55 mL
05707B	NeuroCult™ Chemical Dissociation Solution B	15 mL
05707C	NeuroCult™ Chemical Dissociation Solution C	15 mL

2.2 Equipment

- Lab timer
- Benchtop centrifuge
- Pipette-aid and sterile plastic serological pipettes
- Pipettors: 20 µL, 200 µL, and 1 mL with sterile disposable plastic tips
- Trypan Blue (Catalog #07050)
- Hemocytometer
- Microscope for hemocytometer cell counts
- 15 mL polypropylene conical tubes (Catalog #38009)

2.3 Additional Required Reagents

STEMCELL Technologies recommends culturing mouse neurospheres with NeuroCult™ Proliferation Kit (Mouse & Rat; Catalog #05702) and supplementing with appropriate cytokines. For complete instructions on culturing mouse neurospheres and instructions on the preparation of NeuroCult™ proliferation media, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™, available at www.stemcell.com or contact us to request a copy.

The following reagents are required:

- NeuroCult™ Proliferation Kit (Mouse & Rat; Catalog #05702)
- Human Recombinant EGF (Catalog #78006.1)
- Human Recombinant bFGF (Catalog #78003.1) (only required when using **adult mouse CNS** cells)
- Heparin Solution (Catalog #07980; only required when using **adult mouse CNS** cells)

For additional required reagents when using **rat** or **human** cells, refer to **sections 5.2** and **6.2**, respectively.

3.0 Important Procedure Notes

The following notes are applicable to the procedures in **sections 4.0, 5.0,** and **6.0**.

- **Incubation times** are crucial for performance. It is important to observe the incubation times precisely.
- The **mixing procedure** to resuspend cells involves pipetting the cell suspension up and down gently through the plastic disposable tip in a consistent rhythm *without pressing the plastic disposable tip against the wall of the tube*.
- The entire chemical dissociation procedure should be performed at **room temperature** (15 - 25°C).
- The procedures are for **neurosphere cultures harvested from T-25 cm^2 flasks**. If cultures are harvested from flasks of other sizes, it is important to adjust the volumes accordingly. For instance, when dissociating neurospheres cultured in T-162 cm^2 flasks, the procedure works well when the volumes of NeuroCult™ Chemical Dissociation Solutions A, B, and C are doubled.

4.0 NeuroCult™ Chemical Dissociation of Neurospheres Derived from Embryonic or Adult Mouse CNS Cells

For complete instructions on culturing mouse neurospheres refer to the Technical Manual: In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™, available at www.stemcell.com or contact us to request a copy.

The following procedure is for **neurosphere cultures harvested from T-25 cm^2 flasks**. For other cultureware, adjust volumes accordingly. Perform the procedure at room temperature (15 - 25°C).

1. Observe Day 4 - 7 neurosphere cultures under a microscope to determine if the neurospheres are ready for passaging. If neurospheres are attached to the bottom of the culture flask, tap the flask against a flat surface to detach them. Viable neurospheres should be semi-transparent and phase contrast bright with many of the cells on the outer surface displaying microspikes. Ideally, cultures should contain neurospheres measuring approximately 100 µm or less in diameter. The procedure works less effectively if neurospheres are allowed to grow too large (> 100 µm).
2. Prepare **NeuroCult™ Proliferation Medium without cytokines** and **Complete Embryonic [or Adult] NeuroCult™ Proliferation Medium**. For complete instructions, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™, available at www.stemcell.com or contact us to request a copy.
3. Pre-wet a pipette (e.g. 10 mL serological pipette, Catalog #38004) with **NeuroCult™ Proliferation Medium without cytokines**.

   Note: Wetting the pipette prior to transferring cells prevents cell loss due to cells sticking to the pipette.
4. Using the pre-wetted pipette, remove the culture medium containing suspended neurospheres and transfer to a sterile tissue culture tube (e.g. 15 mL polypropylene conical tube). If some cells remain attached to the culture flask, detach them by pipetting a stream of medium across the attached cells.
5. Centrifuge at 90 x *g* for 5 minutes.
6. Remove and discard as much supernatant as possible, leaving behind a minimal volume (< 50 µL).
7. Add 500 µL **NeuroCult™ Chemical Dissociation Solution A** per cell pellet.
8. Using a 1 mL pipettor set at approximately 450 µL, pipette gently to resuspend neurospheres *without pressing the plastic disposable tip against the wall of the tube*.
9. Set the timer to 8 minutes. Add 125 µL **NeuroCult™ Chemical Dissociation Solution B** to the cell suspension. Gently tap the tube to mix, then immediately start the timer. Incubate at room temperature.

   Refer to Figures 4A and 4B for photomicrographs showing the chemical dissociation of neurospheres at 2 minutes after the addition of NeuroCult™ Chemical Dissociation Solution B.

10. After 3 minutes have elapsed, use a 1 mL pipettor set at approximately 450 µL to gently mix the cell suspension (do not triturate; see section 3.0) by pipetting up and down 8 times. Continue incubation at room temperature.

    Refer to Figures 4C and 4D for photomicrographs showing the chemical dissociation of mouse neurospheres at 3 minutes after addition of NeuroCult™ Chemical Dissociation Solution B.

11. After 7 minutes have elapsed, use a 1 mL pipettor set at approximately 450 µL to pipette the cell suspension gently up and down 8 times. Continue incubation at room temperature.

    Refer to Figures 4E and 4F for photomicrographs showing the chemical dissociation of mouse neurospheres at 7 minutes after addition of NeuroCult™ Chemical Dissociation Solution B.

12. When the timer reaches zero, immediately add 40 µL **NeuroCult™ Chemical Dissociation Solution C**. Mix the cell suspension by pipetting up and down 8 times using a 1 mL pipettor.
13. Add 350 µL **Complete Embryonic [or Adult] NeuroCult™ Proliferation Medium** (prepared in step 2) to bring the final volume to approximately 1 mL. Mix the cell suspension by pipetting up and down. The cell suspension should contain single cells and no obvious aggregates.
14. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.
15. If more than 100 µL of the cell suspension is required for the next subculture in T-25 cm^2 flasks or smaller tissue culture vessels (e.g. wells), wash the cells as described below to remove the chemical reagents:
    a. Add 10 mL NeuroCult™ Proliferation Medium without cytokines to the cell suspension.
    b. Centrifuge at 150 x *g* for 5 minutes.
    c. Gently add 1 mL Complete Embryonic [or Adult] NeuroCult™ Proliferation Medium to resuspend cells. Count viable cells using Trypan Blue and a hemocytometer.
16. Seed 5 x 10^5 cells/10 mL in **Complete Embryonic [or Adult] NeuroCult™ Proliferation Medium**. Refer to Table 1 for volume of medium and number of cells for different flask sizes.

Table 1. Recommended Volume of Medium and Number of Cells for Different Flask Sizes

FLASK SIZE	VOLUME OF MEDIUM	TOTAL CELLS/FLASK	RECOMMENDED FLASK
T-25 cm^2	10 mL	5 x 10^5 cells (or 2 x 10^4 cells/cm^2)	Thermo Fisher Catalog #156367
T-162 cm^2	40 mL	2 x 10^6 cells (or 1.2 x 10^4 cells/cm^2)	Corning Catalog #3151 OR Fisher Scientific Catalog #07-200-64

Note: NeuroCult™ Chemical Dissociation Kit can be used during each subsequent passage for the dissociation of mouse neurospheres to reliably obtain single-cell suspensions for subculturing or other applications.

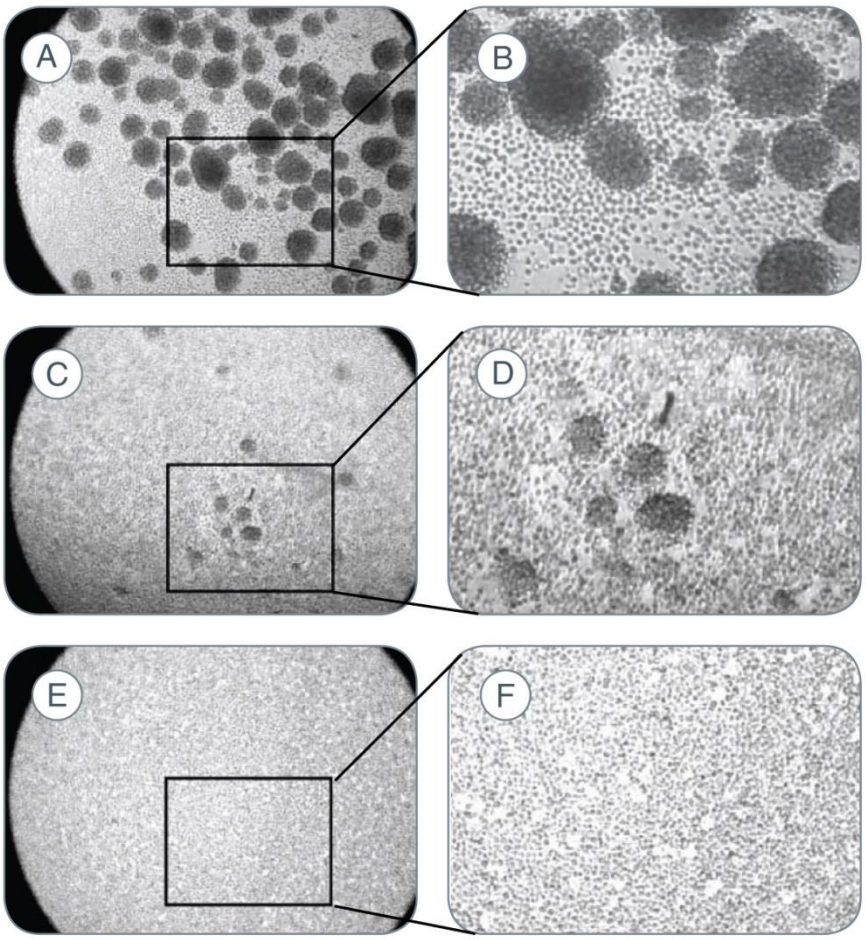

Figure 4. Chemical Dissociation of Mouse Neurospheres
Photomicrographs showing the chemical dissociation of mouse neurospheres at **(A)** 2 minutes, **(C)** 3 minutes, and **(E)** 7 minutes after the addition of NeuroCult™ Chemical Dissociation Solution B. Images **(B)**, **(D)**, and **(F)** are enlargements of the boxed areas in images **(A)**, **(C)**, and **(E)**, respectively. A single-cell suspension is evident in images **(E)** and **(F)**.

5.0 Appendix 1: Dissociation of Neurospheres Derived from Embryonic Day 18 Rat CNS Cells

5.1 Important Notes

- NeuroCult™ Chemical Dissociation Kit **should not be used when functional studies for rat neural stem cells are being performed in long-term rat neurosphere cultures** (> Passage 5). Some adverse effects on stem cell properties have been observed in long-term rat neurosphere cultures when the rat neurospheres were dissociated at each culture passage (Passages 1 - 4) with NeuroCult™ Chemical Dissociation Kit. The adverse effects included a decrease in the ability of long-term self-renewal and lower total cell expansion.
- NeuroCult™ Chemical Dissociation Kit can be used for the dissociation of rat neurospheres when the cells will be used in end-stage applications such as flow cytometric analyses or molecular applications (PCR, microarrays, protein isolation, etc.).
- Use of NeuroCult™ Chemical Dissociation Kit with neurospheres derived from rat cells results in cultures with uniform neurosphere size, high percent viability, and maintenance of total cell expansion between Passages 1 - 5 (Figure 5). Cells from chemically dissociated neurospheres contain a higher percentage of neurons when differentiated compared to cells from triturated neurospheres (Figure 6).
- NeuroCult™ Chemical Dissociation Kit was developed for dissociating neurospheres derived from embryonic and adult mouse CNS cells. The procedure below was modified for the dissociation of neurospheres derived from rat CNS cells.

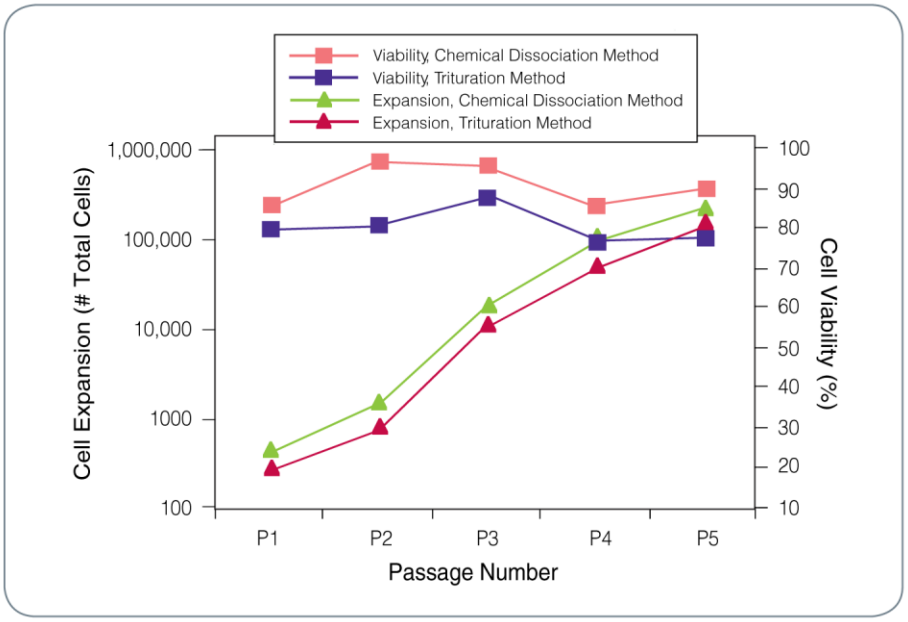

Figure 5. Comparison of Percent Cell Viability and Cell Expansion Between NeuroCult™ Chemical Dissociation Kit and Trituration. Rat neurospheres were dissociated at each passage (up to P5) with NeuroCult™ Chemical Dissociation Kit or trituration. Cells dissociated using NeuroCult™ Chemical Dissociation Kit had a higher percent viability and no adverse effect on total cell expansion between P1 - P5 (n = 3).

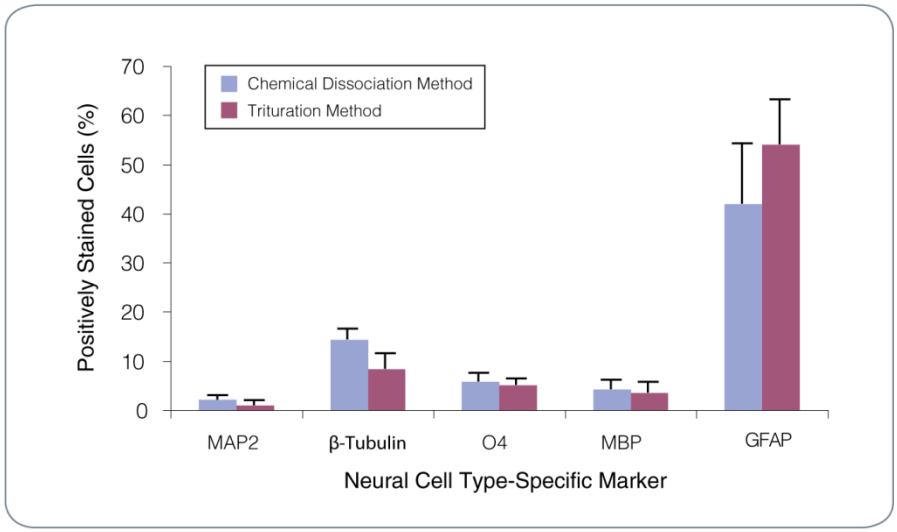

Figure 6. Comparison of the Multi-Lineage Potential of Cells From Rat Neurospheres Dissociated Using NeuroCult™ Chemical Dissociation Kit or Trituration
Cell-specific lineages were determined by immunolabeling with specific antibodies for neurons (Anti-Beta-Tubulin III Antibody, Clone TUJ1, Catalog #60052; anti-MAP2 antibody, clone AP20), oligodendrocytes (Anti-Oligodendrocyte Marker O4 Antibody, Clone 81, Catalog #60053; anti-myelin basic protein [MBP]) and astrocytes (Anti-GFAP Antibody, Polyclonal, Catalog #60128). The percentage of neurons generated by cells dissociated using NeuroCult™ Chemical Dissociation Kit was higher than cells dissociated using trituration.

5.2 Additional Required Reagents

STEMCELL Technologies recommends culturing rat neurospheres using NeuroCult™ Proliferation Kit (Mouse & Rat; Catalog #05702) and supplementing with EGF, bFGF, and Heparin Solution. For complete instructions on culturing rat neurospheres and instructions on the preparation of Complete NeuroCult™ Proliferation Medium, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™, available at www.stemcell.com or contact us to request a copy.

- NeuroCult™ Proliferation Kit (Mouse & Rat; Catalog #05702)
- Human Recombinant EGF (Catalog #78006.1)
- Human Recombinant bFGF (Catalog #78003.1)
- Heparin Solution (Catalog #07980)

5.3 Procedure

The following procedure is for **neurosphere cultures harvested from a single T-25 cm^2 flask**. For other cultureware, adjust volumes accordingly. Perform the procedure at room temperature (15 - 25°C).

1. Observe Day 3 - 4 rat neurosphere cultures under a microscope. Ideally, cultures should contain neurospheres measuring approximately 100 µm in diameter or less. The procedure works less effectively if neurospheres are allowed to grow too large (> 100 µm).
2. Prepare **NeuroCult™ Proliferation Medium without cytokines** and **Complete NeuroCult™ Proliferation Medium**. For complete instructions, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Mouse and Rat Neural Stem and Progenitor Cells Using NeuroCult™, available at www.stemcell.com or contact us to request a copy.

3. Pre-wet a pipette (e.g. 10 mL plastic serological pipette, Catalog #38004) with NeuroCult™ Proliferation Medium **without cytokines**.

   Note: Wetting the pipette prior to transferring cells prevents cell loss due to cells sticking to the pipette.

4. Use the pre-wetted pipette to remove the medium containing suspended rat neurospheres from the culture flask and transfer into a sterile tissue culture tube (e.g. 15 mL polypropylene conical tube).
5. Centrifuge at 90 x *g* for 5 minutes.
6. Remove and discard as much of the supernatant as possible (be careful not to disrupt the pellet), leaving behind a minimal volume (less than 50 µL).
7. Add 250 µL **NeuroCult™ Chemical Dissociation Solution A** per cell pellet. Using a 1 mL pipettor set at approximately 220 µL, pipette gently to resuspend neurospheres *without pressing the plastic disposable tip against the wall of the tube*.
8. For neurospheres < 100 µm in diameter: Set the timer to 6 minutes.

   OR

   For neurospheres > 100 µm in diameter: Set the timer to 7 minutes.

9. Add 72 µL **NeuroCult™ Chemical Dissociation Solution B** to the neurosphere suspension. Gently tap the tube to mix and immediately start the timer.
10. After 3 minutes have elapsed, use a 200 µL pipettor set at 180 µL to gently mix the suspension (do not triturate) by pipetting up and down 8 times.
11. If timer was set to 6 minutes (step 8): After 5 minutes have elapsed from the start of timing, mix the suspension by gently pipetting up and down 8 times.

    OR

    If timer was set to 7 minutes (step 8): After 6 minutes have elapsed from the start of timing, mix the suspension by gently pipetting up and down 8 times.

12. At the end of the 6- or 7-minute incubation period, add 21 µL **NeuroCult™ Chemical Dissociation Solution C** and mix cell suspension by pipetting up and down 8 times. The cell suspension should contain single cells and no obvious aggregates.
13. Wash the cells by adding 10 mL **NeuroCult™ Proliferation Medium without cytokines** to the cell suspension and centrifuge at 150 x *g* for 5 minutes.
14. Remove the supernatant. Using a 1 mL pipettor, gently add 500 µL **Complete NeuroCult™ Proliferation Medium** to resuspend cells.
15. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.
16. Cells are now ready to be cultured or used in subsequent experiments.

6.0 Appendix 2: Dissociation of Neurospheres Derived from Fetal Human CNS Cells

6.1 Important Notes

- The procedure below has been tested using neurospheres derived from cortical cells of an 8- to 14-week-old post-mortem fetus. The procedure has not been standardized for the dissociation of neurospheres derived from different regions of the fetal human CNS.
- In some cases, cells dissociated from human neurospheres using NeuroCult™ Chemical Dissociation Kit tend to lose their stem cell characteristics of long-term self-renewal and high total cell expansion.
- NeuroCult™ Chemical Dissociation Kit is recommended for the generation of a single-cell suspension from neurospheres derived from fetal human CNS cells when the cells will be used in end-stage applications such as flow cytometric analyses or molecular applications (PCR, microarrays, protein isolation, etc).
- NeuroCult™ Chemical Dissociation Kit was developed specifically for dissociating neurospheres derived from embryonic and adult mouse CNS cells. The procedure below has been modified for the dissociation of neurospheres derived from fetal human CNS cells.

6.2 Additional Required Reagents

STEMCELL Technologies recommends culturing human neurospheres with NeuroCult™ NS-A Proliferation Kit (Human; Catalog #05751) or NeuroCult™-XF Proliferation Medium (Catalog #05761) and supplementing with EGF, bFGF, and Heparin Solution. For complete instructions on culturing human neurospheres and instructions on medium preparation refer to the Technical Manual: In Vitro Proliferation and Differentiation of Human Neural Stem and Progenitor Cells Using NeuroCult™ or NeuroCult™-XF, available at www.stemcell.com or contact us to request a copy.

- NeuroCult™ NS-A Proliferation Kit (Human; Catalog #05751) or NeuroCult™-XF Proliferation Medium (Catalog #05761)
- Human Recombinant EGF (Catalog #78006.1)
- Human Recombinant bFGF (Catalog #78003.1)
- Heparin Solution (Catalog #07980)

6.3 Procedure

The following procedure is for **neurosphere cultures harvested from a single T-25 cm^2 flask**. For other cultureware, adjust volumes accordingly. Perform the procedure at room temperature (15 - 25°C).

1. Observe Day 7 - 9 neurosphere cultures of human cells under a microscope. Ideally, cultures should contain neurospheres measuring approximately 100 µm in diameter or less.
2. Prepare **NeuroCult™ Proliferation Medium without cytokines** and **Complete NeuroCult™ Proliferation Medium**. For complete instructions, refer to the Technical Manual: In Vitro Proliferation and Differentiation of Human Neural Stem and Progenitor Cells Using NeuroCult™ or NeuroCult™-XF, available at www.stemcell.com or contact us to request a copy.
3. Pre-wet a pipette (e.g. 10 mL plastic serological pipette, Catalog #38004) with **NeuroCult™ Proliferation Medium without cytokines**.

   Note: Wetting the pipette prior to transferring cells prevents cell loss due to cells sticking to the pipette.
4. Use the pre-wetted pipette to remove the medium containing suspended neurospheres from the culture flask and transfer to a sterile tissue culture tube (e.g. 15 mL polypropylene conical tube).
5. Centrifuge neurospheres at 90 x *g* for 15 minutes.

6. Remove and discard as much supernatant as possible (be careful not to disrupt the pellet), leaving behind a minimal volume (less than 50 µL).
7. Add 250 µL **NeuroCult™ Chemical Dissociation Solution A** per cell pellet. Using a 1 mL pipettor set at approximately 220 µL, gently pipette up and down to resuspend neurospheres *without pressing the plastic disposable tip against the wall of the tube*.
8. Set the timer to 10 minutes. Add 72 µL **NeuroCult™ Chemical Dissociation Solution B** to the neurosphere suspension. Gently mix the suspension by pipetting up and down 10 times and immediately start the timer.
9. After 2, 4, and 6 minutes have elapsed from the start of the timing, use a 200 µL pipettor set at 180 µL to gently mix the suspension by pipetting up and down (do not triturate) 10 times.
10. At the end of the 10-minute incubation period, immediately add 21 µL **NeuroCult™ Chemical Dissociation Solution C** and mix cell suspension by pipetting up and down 10 times. The suspension should contain single cells and no obvious aggregates.
11. Add 10 mL **NeuroCult™ Proliferation Medium without cytokines** to the cell suspension, then centrifuge at 190 x *g* for 10 minutes.
12. Remove the supernatant. Using a 1 mL pipettor, gently add 500 µL **Complete NeuroCult™ Proliferation Medium** to resuspend cells.
13. Measure the total volume of the cell suspension. Count viable cells using Trypan Blue and a hemocytometer.
14. Cells are now ready to be cultured or used in subsequent experiments.

7.0 References

1. Reynolds BA & Weiss S. (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175(1): 1–13.
2. Reynolds BA & Weiss S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052): 1707–10.
3. Sen A et al. (2002) Passaging protocols for mammalian neural stem cells in suspension bioreactors. Biotechnol Prog 18(2): 337–45.

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Chemical Dissociation of Neurospheres Derived From Embryonic and Adult Mouse CNS Tissue Using NeuroCult™ Chemical Dissociation Kit

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