TECHNICAL MANUAL

In Vitro Proliferation and Differentiation of Human Neural Stem and Progenitor Cells Using NeuroCult™ or NeuroCult™-XF

STEMCELL™ TECHNOLOGIES

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* NeuroCult™ media is sold under license from StemCells California, Inc. US Patent Nos. 5,750,376; 5,851,832; 5,980,885; 5,968,829; 5,981,165; 6,071,889; 6,093,531; 6,103,530; 6,165,783; 6,238,922

1.0 Materials and Reagents

1.1 Storage of Basal Media and Supplements

Table 1. NeuroCult™ NS-A Component Storage and Stability

COMPONENT	SIZE	STORAGE AND STABILITY	NOTES
NeuroCult™ NS-A Basal Medium (Human; #05750)	450 mL	Product stable at 2 - 8°C until expiry date (EXP) on label.*	NeuroCult™ NS-A Basal Medium must be used with supplements suitable for the desired cell culture application: NeuroCult™ NS-A Proliferation Supplement and required cytokines or NeuroCult™ NS-A Differentiation Supplement.
NeuroCult™ NS-A Proliferation Supplement (Human; #05753)	50 mL	Product stable at -20°C until expiry date (EXP) on label.* *Storage of aliquots at -20°C is possible; do not freeze/thaw more than twice. Storage at 2 - 8°C is not recommended.*	Components of NeuroCult™ NS-A Proliferation Supplement are pretested and selected to support the growth and expansion of human neural stem and progenitor cells when combined with NeuroCult™ NS-A Basal Medium. Required cytokines are not included.
NeuroCult™ NS-A Differentiation Supplement (Human; #05754)	50 mL	Product stable at -20°C until expiry date (EXP) on label.* *Storage of aliquots at -20°C is possible; do not freeze/thaw more than twice. Storage at 2 - 8° C is not recommended.*	Components of NeuroCult™ NS-A Differentiation Supplement are pretested and selected to support the differentiation of human neural stem and progenitor cells into astrocytes, neurons and oligodendrocytes when combined with NeuroCult™ NS-A Basal Medium.

*After NeuroCult™ NS-A Proliferation or Differentiation Supplement has been added to NeuroCult™ NS-A Basal Medium, store at 2 - 8°C for up to 1 month.

Table 2. NeuroCult™-XF Component Storage and Stability

COMPONENT	SIZE	STORAGE AND STABILITY	NOTES
NeuroCult™-XF Basal Medium (#05760)	450 mL	Product stable at 2 - 8°C until expiry date (EXP) on label.*	NeuroCult™-XF Basal Medium must be used with the NeuroCult™-XF Proliferation Supplement and desired cytokines.
NeuroCult™-XF Proliferation Supplement (#05763)	50 mL	Product stable at -20°C until expiry date (EXP) on label.* *Storage of aliquots at -20°C is possible; do not freeze/thaw more than twice. Storage at 2 - 8°C is not recommended.*	Components of the NeuroCult™-XF Proliferation Supplement are pretested and selected to support the growth and expansion of human neural stem and progenitor cells when combined with NeuroCult™-XF Basal Medium. Required cytokines are not included.

*After NeuroCult™-XF Proliferation Supplement has been added to NeuroCult™-XF Basal Medium, store at 2 - 8°C for up to 1 month.

1.2 Additional Required Reagents and Cultureware

1.2.1 For Proliferation of Neural Stem and Progenitor Cells

- Human Recombinant Epidermal Growth Factor (EGF; 100 µg, Catalog #78006.1)
- Human Recombinant Basic Fibroblast Growth Factor (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)
- Poly-D-Lysine (Sigma Catalog #P7280) [optional; only needed for adherent cultures]
- Laminin (Sigma Catalog #L2020) [optional; only needed for adherent cultures]
- ACCUTASE™ (Catalog #07920) [optional; for passaging cells]
- Trypan Blue (Catalog #07050)

Table 3. Recommended Cultureware for Proliferation (Neurosphere and Adherent Monolayer Methods)

TISSUE CULTURE VESSEL	SUGGESTED SUPPLIERS
6-Well Plate	Corning® Costar® 6-Well TC-Treated Plates (Corning Catalog #3516)
T-25 cm^2 Flask	Nunc™ EasYFlasks™ Nunclon™ (Nunc 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)

1.2.2 For Differentiation of Neural Stem and Progenitor Cells

- 12 mm round glass coverslips (Carolina Biological Catalog #633029) [optional]
- Corning® Matrigel®, Growth Factor Reduced (Corning Catalog #354277 or #354230) [optional]

Table 4. Recommended Cultureware for Differentiation

TISSUE CULTURE VESSEL	SUGGESTED SUPPLIERS
24-Well Plate	Corning® Costar® 24-Well TC-Treated Plates (Corning Catalog #3526)
Corning® Matrigel®-Coated 24-Well Plate	Corning® BioCoat™ Matrigel™ Matrix 24-Well Plates (Corning Catalog #354605)

*Heparin solution contains animal-derived components. To provide a complete xeno-free system, heparin can be omitted.

1.2.3 For 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)
- Neural lineage-specific primary antibodies and secondary detection antibodies (Catalog #05716)

1.3 Required 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
- Micropipettes: 10 µL, 200 µL and 1 mL with sterile disposable plastic pipette tips
- Tubes: 15 mL polypropylene (Corning Catalog #352096) or 17 x 100 mm polystyrene (Corning Catalog #352051)
- Tubes: 50 mL polypropylene (Corning Catalog #352070)
- Hemocytometer
- Forceps for use in moving coverslips during immunolabeling
- Routine light microscope for hemocytometer cell counts
- Inverted microscope with flatfield objectives and eye pieces to give object magnification of approximately 20 - 30X, 80X, and 125X

2.0 Preparation of Materials and Reagents

Use sterile techniques when preparing materials and reagents.

2.1 For Proliferation of Neural Stem and Progenitor Cells

2.1.1 EGF Stock Solution (10 µg/mL)

Note: If a xeno-free preparation of EGF is desired, substitute HSA for BSA in step 1.

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).

3. Store aliquots (0.1 - 0.3 mL) of 10 µg/mL EGF stock solution at -20°C. Do not freeze/thaw each vial more than 3 times.

2.1.2 bFGF Stock Solution (10 µg/mL)

Note: If a xeno-free preparation of bFGF is desired, substitute HSA for BSA in step 1.

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.

2. Store aliquots (0.1 - 0.3 mL) of 10 µg/mL bFGF stock solution at -20°C. Do not freeze/thaw each vial more than 3 times.

2.1.3 Complete NeuroCult™ Proliferation Medium

1. Thaw bottles of NeuroCult™ NS-A Proliferation Supplement (Human) overnight at 2 - 8°C or for 1 - 2 hours at 37°C. NeuroCult™ NS-A Proliferation Supplement can be aliquoted into 10 mL volumes and stored at -20°C until required for use. Repeated thawing and freezing is not recommended.

   Note: If the supplement thaws during shipping, it should be aliquoted immediately. Providing the supplement remains cool (not more than ~10°C), performance should be unaffected.

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

   OR

   Add 1 mL NeuroCult™ NS-A Proliferation Supplement (Human) to each 9 mL of NeuroCult™ NS-A Basal Medium (Human).

3. Mix well. NeuroCult™ Proliferation Medium without cytokines is now ready for use.

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

   After NeuroCult™ NS-A Proliferation Supplement has been added to NeuroCult™ NS-A Basal Medium, store at 2 - 8°C for up to 1 month. Avoid repeated exposure of medium to room temperature and light.

4. To each 10 mL NeuroCult™ Proliferation Medium without cytokines, 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 known hereafter as **Complete NeuroCult™ Proliferation Medium**.

   Note: If not used immediately, store Complete NeuroCult™ Proliferation Medium (cytokine-containing) at 2 - 8°C for up to 1 week.

2.1.4 Complete NeuroCult™-XF Proliferation Medium

1. Thaw bottles of NeuroCult™-XF Proliferation Supplement overnight at 2 - 8°C or for 1 - 2 hours at 37°C. If not used immediately, aliquot into 10 mL volumes and store at -20°C. Avoid repeated freeze-thaw cycles.

   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™-XF Proliferation Supplement to one bottle (450 mL) of NeuroCult™-XF Basal Medium.

   OR

   Add 1 mL NeuroCult™-XF Proliferation Supplement to each 9 mL NeuroCult™-XF Basal Medium.

3. Mix well. NeuroCult™-XF Proliferation Medium without cytokines 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™-XF 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 NeuroCult™-XF Proliferation Medium without cytokines (i.e. NeuroCult™-XF Basal Medium containing NeuroCult™-XF Proliferation Supplement), add the following:
   - 20 µL of xeno-free 10 µg/mL EGF (to give a final concentration of 20 ng/mL EGF)
   - 10 µL of xeno-free 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)

   **Heparin Solution contains non-human animal-derived components and can be omitted if a complete xeno-free system is desired.*

   This will be known hereafter as **Complete NeuroCult™-XF Proliferation Medium**.

   Note: If not used immediately, store Complete NeuroCult™-XF Proliferation Medium (cytokine-containing) at 2 - 8°C for up to 1 week.

2.1.5 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 tissue culture vessel. By coating the surface of the culture vessel with poly-D-lysine (PDL), laminin, poly-L-ornithine, or combinations of these substrates, cell-to-substrate attachment is promoted, as opposed to the cell-to-cell attachment, which leads to aggregation and formation of neurospheres.

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.

Preparation of 10 µg/mL Laminin Stock Solution

1. Thaw laminin at 2 - 8°C, to prevent laminin from gelling.
2. 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).
3. Store the remaining laminin (which has not been diluted) in appropriately-sized working aliquots at -20°C.

Preparation of PDL/Laminin-Coated Tissue 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 tissue culture vessel, as indicated in Table 5.

Table 5. Recommended Substrate Volume for Coating Tissue Cultureware

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

3. Incubate for 2 hours at room temperature (15 – 25°C) or overnight (~20 hours) at 2 - 8°C.
4. Wash each well/flask with sterile PBS, according to the recommended volumes in Table 6. Remove as much of the PBS as possible.

Table 6. Recommended PBS Wash Volume

TISSUE CULTURE VESSEL	VOLUME OF PBS WASH BUFFER
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 5.
6. Incubate for 2 hours at room temperature (15 – 25°C) or overnight at 2 - 8°C.
7. Wash each well/flask with sterile PBS, according to the recommended volumes in Table 6.

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

8. The substrate-coated tissue cultureware is ready for use and should be used within the same day as completing the coating procedure.
9. Proceed to section 4.0 for plating cells for adherent monolayer cultures.

Preparation of Laminin-Only Coated Tissue Cultureware

1. Dispense 10 µg/mL laminin stock solution at the volume indicated in Table 5.
2. Incubate for 2 hours at room temperature (15 – 25°C) or overnight at 2 - 8°C.
3. Wash each well/flask with sterile PBS, according to the recommended volumes in Table 6.

   Note: Only remove the PBS when ready to plate the cells. Do not let the coated plates dry completely.
4. The substrate-coated tissue cultureware is ready for use and should be used within the same day as completing the coating procedure.
5. Proceed to section 4.0 for plating cells for adherent monolayer cultures.

2.2 For Differentiation of Neural Stem and Progenitor Cells

2.2.1 Preparation of Complete NeuroCult™ Differentiation Medium

1. Thaw bottles of NeuroCult™ NS-A Differentiation Supplement (Human) overnight at 2 - 8°C or for 1 - 2 hours at 37°C. If not used immediately, aliquot into 10 mL volumes and store at -20°C. Avoid repeated freeze-thaw cycles.
2. Add the entire volume (50 mL) of NeuroCult™ NS-A Differentiation Supplement to one bottle (450 mL) of NeuroCult™ NS-A Basal Medium (Human).

   OR

   Add 1 mL NeuroCult™ NS-A Differentiation Supplement (Human) to every 9 mL NeuroCult™ NS-A Basal Medium (Human) (1/10 dilution).
3. Mix well. 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.

2.2.2 Suggested Preparation for Xeno-Free Differentiation Medium

1. Add human serum to a final concentration of 2% to NeuroCult™-XF Proliferation Medium without cytokines (refer to section 2.1.4 steps 1 - 3).
2. Mix well. Complete Xeno-Free Differentiation Medium is now ready for use.

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

2.2.3 Coating Cultureware for Differentiation Cultures

If using round glass coverslips for immunocytochemistry, ensure that the chosen coverslips can easily be added to and removed from the wells of the (e.g. 24-well) plate used for culturing the cells. Sterilize coverslips by autoclaving prior to coating with Matrigel®.

Matrigel®-Coated Glass Coverslips

1. 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).

2. 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 of Matrigel® at -20°C.

   Note: One 2 mg Matrigel® aliquot generates enough solution to coat 1 - 2 24-well plates (depending on whether 0.5 or 1 mL of solution is used to coat each well). The protein concentration of each lot of Matrigel® varies. Refer to the supplier Certificate of Analysis to determine the appropriate volume of Matrigel® required to prepare 2 mg aliquots.

3. Add 23 mL NeuroCult™ Proliferation Medium without cytokines (section 2.1.1 steps 1 - 3) into a 50 mL polypropylene tube.
4. Add 1 mL COLD NeuroCult™ Proliferation Medium without cytokines to the tube containing 2 mg Matrigel® and pipette up and down to mix the solution.
5. 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.
6. Using sterile forceps, transfer one pre-sterilized glass coverslip per well of a 24-well plate.
7. Add 0.5 - 1 mL diluted Matrigel® into each well containing a coverslip.
8. Incubate at room temperature (15 - 25°C) for 2 hours or overnight at 2 - 8°C.
9. Remove the Matrigel® solution when ready to use the coated glass coverslips.

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

10. Proceed to section 5.0 for plating cells for differentiation.

3.0 Expansion of Neural Stem and Progenitor Cells in Neurosphere Cultures

3.1 Background

Neural stem cells (NSCs) can be isolated from different regions of the human embryonic and fetal CNS, including the striatum, cortex, spinal cord, thalamus, and ventral mesencephalon[1] at different stages of development. In the adult human brain NSCs reside within specific niches, such as the subventricular zone of the lateral ventricle.[2] Multipotent NSC-like cells, known as brain tumor stem cells (BTSCs) or cancer stem cells (CSCs), have been identified and isolated from different grades and types of brain cancers, including gliomas, medulloblastomas, astrocytomas, and ependymomas.[3] For information on the isolation of primary human CNS tissues, refer to "Cultures of Stem Cells from the Central Nervous System."[4] All procedures must be performed in accordance with rules outlined by local ethics committees. The protocol below describes a method for expansion of human NSCs from primary tissues (obtained using standard microdissection techniques) in neurosphere cultures.

NeuroCult™ NS-A and NeuroCult™-XF (xeno-free) Proliferation Media are optimized for the culture of human neural stem and progenitor cells as cellular aggregates called neurospheres.

Important notes when culturing human neurospheres:

1. Overall, human NSCs have a slower growth rate than mouse NSCs; however, the growth rate is also highly dependent on the CNS region from which the cells are isolated. In optimized culture conditions human embryonic CNS cells (e.g. forebrain regions: telencephalon, diencephalon) exhibit a doubling time between 5 - 12 days, whereas mouse embryonic CNS cells only require 1 - 2 days.
2. Expansion of human embryonic CNS stem cells requires the presence of both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).
3. Human neural stem cells are extremely sensitive to mechanical dissociation. To avoid excessive cell death, observe the following precautions and follow the Trituration procedure in section 3.3, step 4.
   - Do not allow neurospheres to overgrow as it is more difficult to dissociate larger neurospheres. Neurospheres should not exceed 100 µm in diameter.
   - Always pre-wet the pipette tip with medium before drawing cells up, to prevent the cells from sticking to the walls of the tip.

3.2 Initial Plating of Primary Human CNS Cells in Neurosphere Cultures

1. Once CNS tissue has been separated from other tissues, dissected and processed to create a single-cell suspension, resuspend the cells in 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium in a 15 mL polypropylene tube or 17 x 100 mm polystyrene tube.

   Note: Use of polypropylene tubes is a precautionary step to prevent cell loss due to cells sticking to the plasticware.
2. Using a pre-wet disposable pipette tip attached to a 1 mL micropipette set at 0.99 mL, triturate the cell sample using a consistent rhythm approximately 20 - 30 times, or until a single-cell suspension is achieved.

   Note: It is extremely important not to expel the last volume of medium from the tip as this will introduce air bubbles into the cell suspension, which will damage or kill cells.
3. Add 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium to the single-cell suspension and mix carefully to avoid creating any bubbles. Leave for approximately 1 minute to allow any undispersed pieces of tissue to settle.
4. Transfer the supernatant containing the single-cell suspension to a new sterile 15 mL tube.

5. Centrifuge at 192 x *g* for 10 minutes. Discard the supernatant.
6. Add 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium and resuspend cells with a brief trituration (pipette up and down 2 times) using a 1 mL micropipette set at 0.99 mL.
7. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.
8. Seed cells at a density of 5 x 10^4 viable cells/cm^2 in Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium as indicated in Table 7.

Table 7. Recommended Primary Human CNS Cell Plating Conditions for Neurosphere Cultures

TISSUE CULTURE VESSEL	VOLUME OF MEDIUM	CELL DENSITY	TOTAL CELLS
6-well plate	3 mL/well	5 x 10^4 viable cells/cm^2	4.75 x 10^5 cells
T-25 cm^2 flask	6 mL	5 x 10^4 viable cells/cm^2	1.25 x 10^6 cells
T-75 cm^2 flask	10 mL	5 x 10^4 viable cells/cm^2	3.75 x 10^6 cells

9. Incubate cultures at 37°C in a 5% CO_2 humidified incubator.
10. Monitor the condition of the cultures daily (e.g. morphology of neurospheres, cell density, media depletion, etc.). Viable neurospheres generally appear semi-transparent and phase bright, with many of the cells on the outer surface displaying microspikes. Refer to section 7.1 for representative images of cultured neurospheres.
11. Replenish the medium every 2 days until the cells are ready for subculture. To replenish the medium, add 0.5 mL (T-25 cm^2) or 1 mL (T-75 cm^2) fresh Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium. Do not exceed the volume capacity of the flasks (i.e. 11 mL for T-25 cm^2 flask or 21 mL for T-75 cm^2 flask).

3.3 Passaging Cells from Neurosphere Cultures

Neurospheres should be passaged when they reach approximately 100 - 150 µm in diameter (typically occurs 7 - 14 days after plating, although in some cases it may take up to 21 days), to avoid generating hypoxic cells in the centre of the spheres. 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 the medium does turn orange/yellow in color before the neurospheres have reached 100 - 150 µm in diameter, it is recommended to 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, becoming necrotic.

1. Harvest and collect the entire cell suspension from the culture into a 50 mL tube (if using a T-75 cm^2 or T-162 cm^2 flask), or a 15 mL tube (if using a T-25 cm^2 flask), depending on the volume harvested. If neurospheres are attached to the culture flask, tap the culture flask or shoot a stream of medium across the attached cells to detach them. Pool all of the cells and medium into the same tube.

   Note: Use of polypropylene is precautionary, to prevent cell loss due to cells sticking to the plasticware.
2. Centrifuge at 192 x *g* for 10 minutes.
3. Remove supernatant, leaving behind approximately 0.2 mL (200 µL) medium.
4. Triturate neurospheres with a 200 µL micropipette as follows:
   a) Adjust the volume on the 200 µL micropipette to 180 µL, to avoid expelling the entire cell suspension and producing bubbles. Setting the pipette to a volume less than 180 µL will not provide efficient

mechanical force to dissociate all of the neurospheres. Resuspend cells in a maximum volume of 200 µL.

b) Angle the tube and pipette tip at 45°, however, do not press the tip against the side or bottom of the tube as this will create too much force on the cells during expulsion. Triturate the neurospheres by pipetting up and down 50 - 100 times in a firm, but not too vigorous, consistent rhythm.

c) Rinse the side of the tube during trituration to remove the remaining neurospheres that are attached to the side of the tube; however, do not rinse too high above the cell suspension volume.

d) Once the cells appear to be in a single-cell suspension (i.e. no obvious cell aggregates detected by eye), observe an aliquot of the cell suspension on a hemocytometer, to determine if any cellular aggregates remain.

e) Repeat the trituration procedure an additional 25 - 35 times, if needed, to dissociate cellular aggregates. A homogenous single-cell suspension should be present at this point.

5. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.
6. Seed cells at a density of 1 x 10^4 viable cells/cm^2 in Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium as indicated in Table 8.

Table 8. Recommended Subculture Plating Conditions for Neurosphere Cultures

TISSUE CULTURE VESSEL	VOLUME OF MEDIUM	CELL DENSITY	TOTAL CELLS
6-well plate	3 mL/well	1 x 10^4 viable cells/cm^2	9.5 x 10^4 cells
T-25 cm^2 flask	6 mL	1 x 10^4 viable cells/cm^2	2.5 x 10^5 cells
T-75 cm^2 flask	10 mL	1 x 10^4 viable cells/cm^2	7.5 x 10^5 cells

7. Incubate cultures at 37°C in a 5% CO_2 humidified incubator.
8. Monitor cultures daily. Medium should be replenished every 2 days after plating, as described in section 3.2 step 11.

Note: Depending on the culture conditions and the CNS region from which the cells were obtained, a 2-fold or greater expansion of total cells should be observed by the end of the culture period.[1]

4.0 Expansion of Neural Stem and Progenitor Cells in Adherent Monolayer Cultures

4.1 Background

Neural stem cells (NSCs) can be isolated from different regions of the human embryonic and fetal CNS including the striatum, cortex, spinal cord, thalamus, and ventral mesencephalon[1] at different stages of development. In the adult human brain NSCs reside within specific niches, such as the subventricular zone of the lateral ventricle.[2] Multipotent NSC-like cells, known as brain tumor stem cells (BTSCs) or cancer stem cells (CSCs), have been identified and isolated from different grades and types of brain cancers, including gliomas, medulloblastomas, astrocytomas, and ependymomas.[3] For information on the isolation of primary human CNS tissues refer to "Cultures of Stem Cells from the Central Nervous System."[4] All procedures must be performed in accordance with rules outlined by local ethics committees. The protocol below describes a method for expansion of human NSCs from primary tissues (obtained using standard microdissection techniques) in adherent monolayer cultures.

4.2 Initial Plating of Primary Human CNS Cells in Adherent Monolayer Cultures

1. Once CNS tissue has been separated from other tissues, dissected and processed to create a single-cell suspension, resuspend the cells in 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium in a 15 mL polypropylene tube or 17 x 100 mm polystyrene tube.

   Note: Use of polypropylene is precautionary, to prevent cell loss due to cells sticking to the plasticware.

2. Using a pre-wet disposable pipette tip attached to a 1 mL micropipette set at 0.99 mL, triturate the cell sample using a consistent rhythm approximately 20 - 30 times, or until a single-cell suspension is achieved.

   Note: It is extremely important not to expel the last volume of medium from the tip as this will introduce air bubbles into the cell suspension, which will damage or kill cells.

3. Add 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium to the single-cell suspension and mix carefully to avoid creating any bubbles. Leave for approximately 1 minute to allow any undispersed pieces of tissue to settle.
4. Transfer supernatant containing the single-cell suspension to a new sterile 15 mL tube.
5. Centrifuge at 192 x *g* for 10 minutes. Discard the supernatant.
6. Add 1 mL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium and resuspend cells with a brief trituration (pipette up and down 2 times) using a 1 mL micropipette set at 0.99 mL.
7. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.
8. Seed cells at a density of 5 x 10^4 viable cells/cm^2 in Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium into **tissue culture vessels coated with PDL/laminin or laminin** as indicated in Table 9. For coating instructions see section 2.1.5.

Table 9. Recommended Primary Human CNS Cell Plating Conditions for Adherent Cultures

TISSUE CULTURE VESSEL	VOLUME OF MEDIUM	CELL DENSITY	TOTAL CELLS
6-well plate	3 mL/well	5 x 10^4 viable cells/cm^2	4.75 x 10^5 cells
T-25 cm^2 flask	10 mL	5 x 10^4 viable cells/cm^2	1.25 x 10^6 cells

9. Incubate cultures at 37°C in a 5% CO_2 humidified incubator.

   Note: In the presence of a substrate, neural stem and progenitor cells will adhere to the substrate-coated tissue culture vessel within 24 hours. The attached cells show a flattened morphology and are mostly bipolar.

4.3 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 pre-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 dissociated cells with the ability to initiate new adherent cultures for multiple passages.

1. Remove the medium from the culture vessel using a pipette.
2. Add PBS to wash the cells as follows:
   - To each well of a 6-well plate, add 3 mL PBS
   - To each T-25 cm^2 flask, add 10 mL PBS
3. Swirl the culture plate or flask gently and then remove the PBS and discard.
4. Add ACCUTASE™ as follows:
   - To each well of a 6-well plate, add 0.5 mL ACCUTASE™
   - To each T-25 cm^2 flask, add 1 mL ACCUTASE™
5. Incubate for 5 minutes at room temperature (15 – 25°C) or at 37°C.
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, shoot a stream of medium across the surface of the tissue culture vessel to detach the cells (refer to steps 7 and 8 below).
7. Add Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium using a disposable pipette to the detached cells as follows:
   - To each well of a 6-well plate, add 2 mL medium
   - To each T-25 cm^2 flask, add 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 tube. If cells remain in the vessel, add a small volume of Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium and repeat the procedure to collect the remaining cells.

   Note: Use of polypropylene is precautionary, to prevent cell loss due to cells sticking to the plasticware.

10. Centrifuge at 110 x *g* for 5 minutes.
11. Remove the supernatant and resuspend cells in a maximum of 200 µL Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium with a plastic disposable pipette tip attached to a 200 µL micropipette set at 180 µL, pipetting until a single-cell suspension is achieved.
12. Resuspend cells in an appropriate (approximately 0.5 - 1 mL) volume of Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium.
13. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.
14. Seed cells at a density of 1 x 10^4 viable cells/cm^2 in Complete NeuroCult™ Proliferation Medium or Complete NeuroCult™-XF Proliferation Medium into **tissue culture vessels coated with PDL/laminin or laminin** as indicated in Table 10. For coating instructions see section 2.1.5.

Table 10. Recommended Subculture Plating Conditions for Adherent Cultures

TISSUE CULTURE VESSEL	VOLUME OF MEDIUM	CELL DENSITY	TOTAL CELLS
6-well plate	3 mL/well	1 x 10^4 viable cells/cm^2	9.5 x 10^4 cells
T-25 cm^2 flask	10 mL	1 x 10^4 viable cells/cm^2	2.5 x 10^5 cells

15. Incubate cultures at 37°C in a 5% CO_2 humidified incubator. This procedure can be repeated for multiple passages.

5.0 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 the cytokines and addition of a small amount of serum, neural stem and progenitor cells are induced to differentiate into neurons, astrocytes and oligodendrocytes.

5.1 Harvesting Cells from Neurosphere Cultures for Differentiation

To initiate differentiation, single cells obtained from dissociated neurospheres should be cultured on Matrigel®-coated round glass coverslips in 24-well plates

Neurospheres should be harvested 7 - 10 days after plating. At this stage the neurospheres will be approximately 100 µm in diameter.

Note: Before harvesting the cells, it is important to prepare the required number of pre-coated coverslips needed for subculture.

1. Collect neurospheres and place in a sterile 15 mL polypropylene tube or 17 x 100 mm polystyrene tube.

   Note: Use of polypropylene is precautionary, to prevent cell loss due to cells sticking to the plasticware.
2. Centrifuge at 192 x *g* for 10 minutes.
3. 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 or Complete Xeno-Free Differentiation Medium using a 10 mL disposable plastic pipette.
5. Centrifuge at 192 x *g* for 10 minutes.
6. Remove supernatant, leaving behind approximately 200 µL of the medium. Using a 200 µL micropipette set at 180 µL, triturate the neurosphere suspension (according to the instructions in section 3.3, step 4) until a single-cell suspension is achieved.
7. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.

5.2 Harvesting Cells from Adherent Monolayer Cultures for Differentiation

Cells should be harvested when the culture is 60 - 80% confluent.

To initiate differentiation, single cells obtained from adherent monolayer cultures should be cultured on Matrigel®-coated round glass coverslips in 24-well plates.

Note: Before harvesting the cells, it is important to prepare the required number of pre-coated coverslips needed for subculture.

1. Remove the medium from the culture vessel using a pipette.
2. Add PBS to wash the cells as follows:
   - To each well of a 6-well plate, add 3 mL PBS
   - To each T-25 cm^2 flask, add 10 mL PBS
3. Swirl the culture plate or flask gently and then remove the PBS and discard.
4. Add ACCUTASE™ as follows:
   - To each well of a 6-well plate, add 0.5 mL ACCUTASE™
   - To each T-25 cm^2 flask, add 1 mL ACCUTASE™

5. Incubate for 5 minutes at room temperature (15 – 25°C) or at 37°C.
6. Observe the culture to determine if detachment is complete. If cells have not completely detached after 5 minutes, shoot a stream of medium across the surface of the tissue culture vessel to detach the cells (refer to steps 7 and 8 below).
7. Wash the cells to remove the cytokines by adding Complete NeuroCult™ Differentiation Medium or Complete Xeno-Free Differentiation Medium. Using a disposable pipette add medium to the detached cells as follows:
   - To each well of a 6-well plate, add 2 mL medium
   - To each T-25 cm^2 flask, add 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 tube. If cells remain in the vessel, add a small volume of Complete NeuroCult™ Differentiation Medium or Complete Xeno-Free Differentiation Medium and repeat the procedure to collect the remaining cells.

   Note: Use of polypropylene is precautionary, to prevent cell loss due to cells sticking to the plasticware.
10. Centrifuge at 110 x *g* for 5 minutes.
11. Remove the supernatant and resuspend cells in a maximum of 200 µL Complete NeuroCult™ Differentiation Medium or Complete Xeno-Free Differentiation Medium with a plastic disposable pipette tip attached to a 200 µL micropipette 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 NeuroCult™ Differentiation Medium or Complete Xeno-Free Differentiation Medium.
13. Measure the total volume of the cell suspension. Count viable cells using trypan blue dye exclusion and a hemocytometer.

5.3 Plating Cells for Differentiation

1. Resuspend cells in Complete NeuroCult™ Differentiation Medium or Complete Xeno-Free Differentiation Medium to yield a plating cell density of 4.2 - 5.3 x 10^4 cells/cm^2.
2. Seed cells onto Matrigel®-coated round glass coverslips in 24-well plates or onto Matrigel®-coated plates as indicated in Table 11.

Table 11. Recommended Plating Conditions for Differentiation

TISSUE CULTURE VESSEL	VOLUME OF MEDIUM	CELL DENSITY	TOTAL CELLS
24-well plate with Matrigel®-coated glass coverslips	1 mL	4.2 - 5.3 x 10^4 cells/cm^2	0.8 - 1 x 10^5 cells
Matrigel®-coated 24-well plate	1 mL	4.2 - 5.3 x 10^4 cells/cm^2	0.8 - 1 x 10^5 cells

3. Incubate cultures in a 5% CO_2 humidified incubator at 37°C.
4. Monitor 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 or Complete Xeno-Free Differentiation Medium.
5. Cultures should be examined after 5 - 10 days with an inverted light microscope to determine if the cells have differentiated (attached). Viable cells appear phase contrast bright. If differentiated cells are present, cells can be processed for indirect immunofluorescence as described in section 6.0.

6.0 Immunolabeling to Identify Differentiated Cell Types

6.1 Fixation

1. Remove approximately 90% of the culture medium from each well of a 24-well plate.

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

6.2 Permeabilization

1. Add 1 mL of 0.3% Triton X-100 (in PBS) to each well.
2. Incubate for 5 - 10 minutes at room temperature (15 - 25°C).
3. Remove Triton X-100/PBS.
4. Add 1 mL PBS (pH 7.2) to each well and incubate for 5 minutes at room temperature (15 - 25°C). Remove PBS.
5. Repeat the PBS wash procedure 2 more times for a total of 3 washes.

6.3 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 volume of 250 µL should be added to each well.*

   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 desired wells of a 24-well plate.
4. Incubate for 2 hours at 37°C or overnight at 2 - 8°C.
5. Wash off the primary antibodies with 3 x 5-minute PBS washes.

6.4 Secondary Labeling

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

   Note: Fluorophore-conjugated secondary antibodies are sensitive to light; keep samples in the dark to prevent bleaching.

* 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.

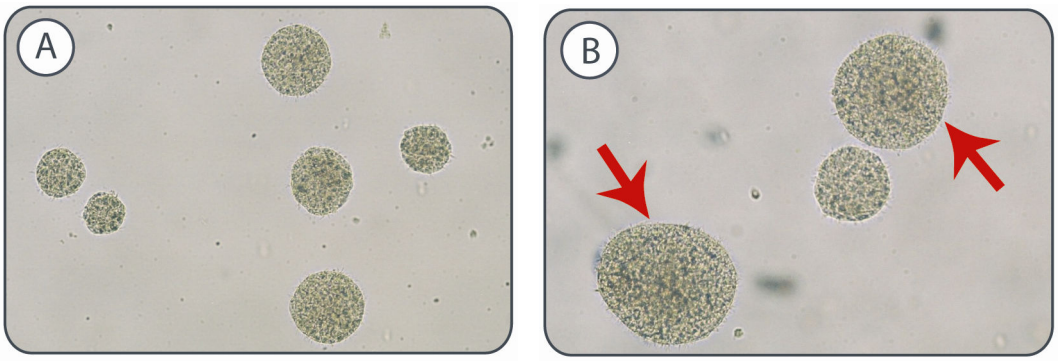
3. Wash off the secondary antibody with 3 x 5-minute PBS washes.
4. After the last wash, add distilled water to each well.

6.5 Mounting

1. Add 10 µL mounting medium (e.g. FluorSave™ Reagent, EMD Millipore Catalog #345789) onto a clean glass coverslip. Remove immunolabeled coverslip from the 24-well plate and gently tap corner of the coverslip to remove excess water.
2. Place coverslip cells-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. Refer to section 8.0 for representative images of immunolabeled differentiated cells.

7.0 Representative Images of Neural Stem and Progenitor Cell Cultures

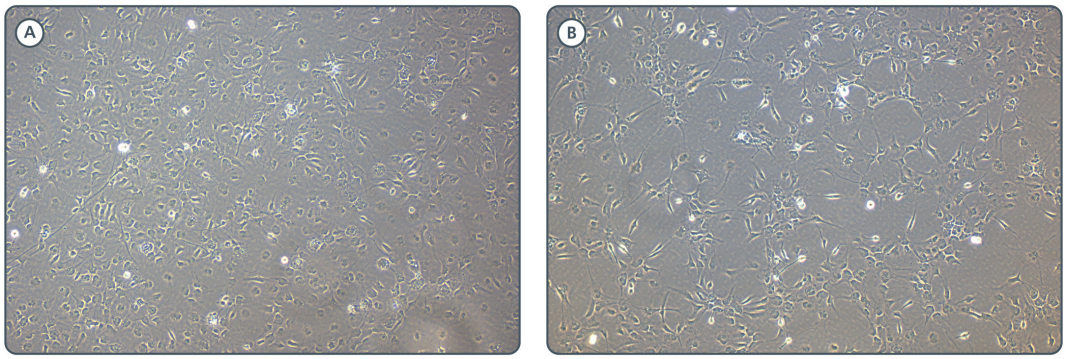
7.1 Neurosphere Cultures



Figures A - B. Neurospheres derived from human CNS cells observed after being cultured for 10 days in Complete NeuroCult™ Proliferation Medium. Neurospheres measure approximately 100 - 200 µm in diameter, are phase contrast bright, semi-transparent and have small microspikes on the periphery of the spheres (arrows).
Magnification: 10X (A) and 40X (B).

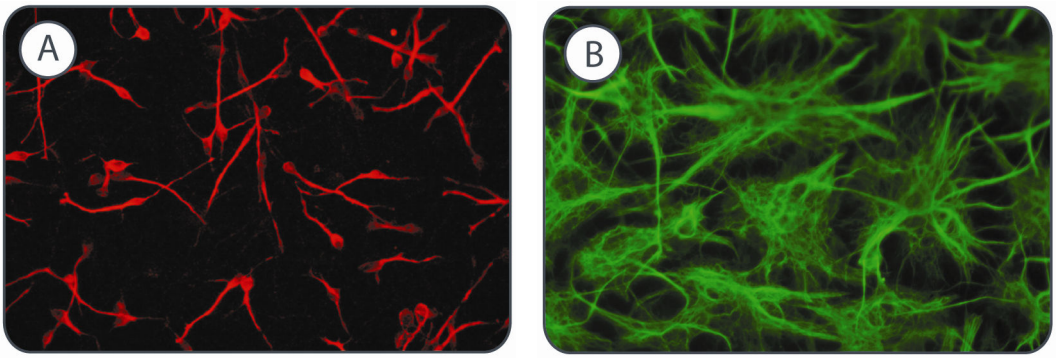
Note: Human CNS-derived neurospheres cultured in NeuroCult™-XF Proliferation Medium display similar morphology.

7.2 Adherent Monolayer Cultures



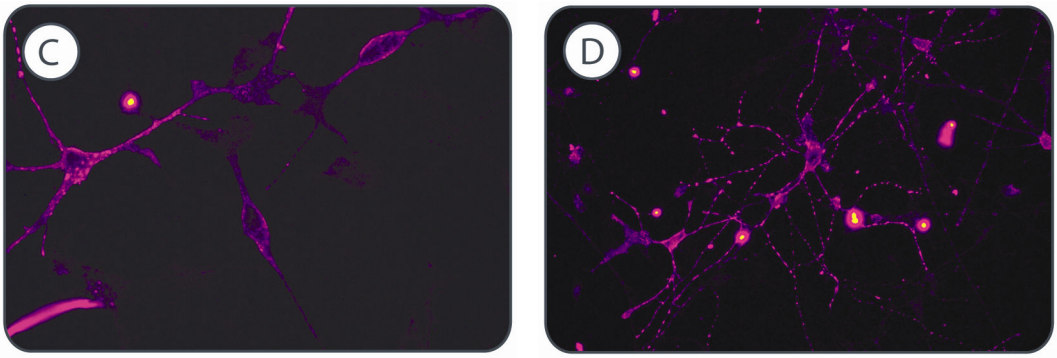
Figures A - B. Adherent monolayer cultures of human CNS-derived neural stem and progenitor cells cultured in Complete NeuroCult™ Proliferation Medium on tissue culture plates coated with 10 µg/mL laminin. A) Day 10 of in vitro culture of passage 10 cells (~80 - 85% confluent). B) Day 16 of in vitro culture of passage 11 cells (~60% confluent).
Magnification: 10X.

8.0 Representative Images of Immunofluorescent Staining to Identify Differentiated Cells Obtained from Neurospheres



A. Neurons (red) were detected with a mouse monoclonal β-tubulin III antibody. Magnification: 10X.

B. Astrocytes (green) were detected with a rabbit polyclonal GFAP antibody. Magnification: 20X.



C. Immature oligodendrocytes (purple) were detected with a rabbit monoclonal O4 Oligodendrocyte Marker antibody. Magnification: 20X.

D. Mature oligodendrocytes (purple) were detected with a galactocerebroside antibody. Magnification: 20X.

Figures A - D. Neurospheres derived from human CNS tissue were cultured in NeuroCult™ Proliferation Medium. Prior to initiating differentiation, neurospheres were dissociated and plated on coverslips. NeuroCult™ Differentiation Medium was used to promote differentiation of neural stem and progenitor cells to neurons, astrocytes and oligodendrocytes.

Note: Cells obtained from neurospheres cultured in NeuroCult™-XF Proliferation Medium and differentiated under xeno-free differentiation conditions display similar immunofluorescent staining patterns when treated with the same antibodies.

Images courtesy of A. Vescovi.

9.0 References

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