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Methods for Growth and Differentiation of Human Neural Stem Cells

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
2. The neural precursor cells can be maintained with an antibiotic if required in order to prevent bacterial contamination. All our lines have been tested with gentamycin (30μg/ml).
3. Incubator should be low oxygen capable such as the NuAire 4950 with the following settings
   a. 6% CO_2
   b. 2-4% O_2
   c. 95% humidity
   d. 37°C

   NOTE: It is best to culture neural cells in low oxygen conditions for optimal growth of hNSCs and differentiation into neurons. Atmospheric oxygen incubators may be used but will result in slower growth of hNSCs and differentiated neural cultures with lower percentage of neurons and high percentage of astrocytes and oligodendrocytes.

II. Required Supplies

1. Neural StemCell Growth Medium (Cat.# 21001-250)
   a. Contains Neural StemCell Growth Basal Medium and Neural StemCell Growth Supplement Pack containing the following components:

Supplement Pack Components
Neural StemCell Growth Base Supplement (contains glutamine)
Laminin
bFGF
EGF
Proprietary Factor

2. Neural Transition Medium to prepare NSCs for differentiation (Cat.# 21003-250)
   a. Contains Neural Transition Basal Medium and Neural Transition Supplement Pack containing the following components:

Supplement Pack Components
Neural Transition Base Supplement (contains glutamine)
Laminin
bFGF
EGF

3. Neural Differentiation Medium for pan-neural differentiation (Cat.# 21004-250)
   a. Contains Neural Differentiation Basal Medium and Neural Differentiation Supplement Pack containing the following components:

Supplement Component
Neural Differentiation Base Supplement (contains glutamine)
Laminin
BDNF
GDNF

4. 0.05% Trypsin EDTA (Cat.# 41004-100)
5. 0.25% Soybean Trypsin Inhibitor (Cat.# 41005-100)
6. D-PBS with calcium and magnesium (Cat.# 41001-500)
7. Laminin coated plates (e.g BD Laminin Biocoat plates or see method below)
8. Poly-d-lysine coated plates (e.g. BD 96-well Biocoat 354640 or 384-well Biocoat 354836 or see method below)
9. Neural StemCell Freezing medium for NSCs (Catalog # 21005-050)

Coating Plates with Poly-D-Lysine

1. Make Poly-D-Lysine (Sigma P7280) solution at 20ug/ml (if using PDL alone) or 5ug/ml (for PDL/Laminin coating) in cell culture grade water and filter through 0.2µm filter.
2. Add appropriate volume (refer to Table 1) of Poly-D-Lysine solution per cell culture vessel and let set in hood for at least 5 minutes.
3. Remove Poly-D-Lysine solution and wash with sterile cell culture grade water (keep lid on plate to avoid contamination). PDL Plates are stable and can be stored at 4-8ºC for up to 2 years.

Coating Plates with Laminin

1. Make laminin (Sigma L-2020 or Life Technologies 23017-015) solution at 10ug/ml in a balanced salt solution then filter through a 0.2µm filter. There is much variability in Laminin sources as well as LOT to LOT variability so make sure the laminin you purchase is optimal for your cells. If the laminin is not optimal, the cells will look clumped or may appear to be coming off the plate. If you observe this try another laminin source. We suggest testing a vial and if it works purchasing several vials to serve as your laminin stock.
2. To each PDL dish/plate, add appropriate volume (refer to Table 1) of laminin solution per cell culture vessel and Let set in hood for 2 hours.
3. Remove laminin and use immediately or place in sterile bag and store at 4-8ºC for up to three months.

Table 1. Tissue Culture Dishes and Flasks Volume for Coating Plates

Dish/Flask Size	Growth Area (cm^2)	Coating Volume
100mm	58.1	5ml
60mm	21.3	3ml
35mm	9.6	1.5ml
6-Well	9.6	1.5ml
12-Well	3.8	300ul
4-Well	2.0	200ul
24-Well	2.0	200ul
48-Well	1.1	100ul
96-Well	.32	50ul
384-Well	.136	30ul
2-Chamber	4.2	300ul
4-Chamber	1.8	200ul
8-Chamber	0.8	100ul

III. Safety Working with Human Cells

1. Assume that all cells isolated from human tissue are potentially hazardous even though they test negative for human pathogens
2. Always wear personal protective equipment
    a. Wear appropriate lab coat, gloves and safety glasses
3. All operations should be conducted using aseptic technique within a biosafety cabinet

IV. Preparing Neural StemCell Growth Medium

1. Warm Neural StemCell Growth basal medium to room temperature
2. Thaw Neural StemCell Growth Supplement and growth factors
3. Aseptically add the supplement then growth factors to the basal medium.
4. Antibiotics are optional but may be added. The cells have been tested in media containing 150ul of Gentamicin (Gibco Cat. # 15750-060 50mg/ml) in 250ml media.
5. Filter through 0.22µm filter unit
6. Store the complete medium at 4°C in the dark until ready for use
7. Warm the complete medium to room temperature immediately before use.
    a. Do not leave the complete medium in a water-bath for longer than 5 minutes as this may result in degradation of the growth factors
8. Use Neural StemCell Growth complete medium within two weeks

V. Preparing Neural Transition Medium

1. Warm Neural Transition basal medium to room temperature
2. Thaw Neural Transition Supplement and growth factors
3. Aseptically add the supplement then growth factor to the basal medium.
4. Antibiotics are optional but may be added. The cells have been tested in media containing 150ul of Gentamicin (Gibco Cat. # 15750-060 50mg/ml) in 250ml media.
5. Filter through 0.22µm filter unit
6. Store the complete medium at 4°C in the dark until ready for use
7. Warm the complete medium to room temperature immediately before use.
    a. Do not leave the complete medium in a water-bath for longer than 5 minutes as this may result in degradation of the growth factors
8. Use Neural Transition complete medium within two weeks

V1. Preparing Neural Differentiation Pan-Neural Differentiation Medium

1. Warm Neural Differentiation basal medium to room temperature
2. Thaw Neural Differentiation Supplement, laminin and growth factors
3. Aseptically add the supplement, laminin and growth factors to the basal medium – rinse each vial to ensure the full contents are transferred to the medium.
4. Antibiotics are optional but may be added. The cells have been tested in media containing 150ul of Gentamicin (Gibco Cat. # 15750-060 50mg/ml) in 250ml media.
5. Filter through 0.22µm filter unit
6. Store Neural Differentiation complete medium at 4°C in the dark until ready for use
7. Warm Neural Differentiation medium to room temperature immediately before use.
    a. Do not leave the Neural Differentiation complete medium in a water-bath for longer than 5 minutes as this may result in degradation of the growth factors
8. Use Neural Differentiation complete medium within two weeks

VII. Thawing Human NSCs

1. Thaw cells at the end of the day as late as possible.
2. Warm the water-bath to 37°C.
3. Prepare the laminin coated plate to receive cells
    a. Add 12ml Neural StemCell Growth complete medium to the laminin coated plate
    e. Place the plate in the humidified incubator set at 6% CO_2, 5% O_2, 37°C
    b. Allow the media in the plates to equilibrate for 1 hour in the incubator prior to thawing the cells.
4. To thaw the cells, remove the vial of cells from the LN_2 freezer and take it immediately to the water-bath. If the freezer is not in close proximity to the water-bath, transport the vial of cells in dry ice.
5. Holding onto the cap, submerge the vial into the water continuously moving the vial in the water to ensure the water surrounding the vial remains warm.

6. Every 10-15 seconds remove the vial from the water and gently turn upside down to see if the frozen cell suspension has thawed. When the cell suspension becomes a slurry take to the safety cabinet
7. Douse the vial in alcohol dry and place inside the safety cabinet.
8. Remove the equilibrated plate from the incubator and place inside the safety cabinet
9. Open the vial and using a 1-2ml pipette gently remove the cell suspension from the vial.
10. Transfer the cell suspension into the media in the equilibrated plate. Make sure to submerge the tip of the pipette below the surface of the media and gently express the cell suspension into the media.
11. Wash the vial with 1ml media from the plate and add back to the plate
12. Rock the plate back and forth, front to back to evenly distribute the cells in suspension
13. Take an aliquot for cell counting to determine percent viability
    a. Take 20ul cell suspension and add it to 20ul Trypan Blue. This provides a dilution factor of 2.
    b. Pipette up and down
    c. Take 10ul and add to the counting chamber of a hemacytometer
    d. Count the live cells and then count the blue (dead) cells within the center grid

Cell count X 10,000 X 2 X Volume = Total cell count

10,000 is a constant for the surface area of the counting chamber
2 is the dilution factor
Volume is the volume of the cell suspension being counted

14. Calculate the total cell count for each the live cells and the dead cells
15. To determine percent live cells
    a. Total live cell count + total dead cell count = total cells
    b. Total Live cell count ÷ total cells x 100 = percent live cells

16. Transfer the plate back into the incubator rocking it side to side, front to back to distribute the cells evenly in the plate.
17. First thing the next morning, remove the media from the cells and replace with 12ml of fresh Neural StemCell Growth complete medium.

VIII. Feeding hNSCs

1. hNSCs should be fed every other day for optimal growth.
2. Warm Neural StemCell Growth complete media to at least room temperature. If warming in water-bath, don't leave in water-bath longer than 5 minutes, as proteins and growth factors easily degrade when left in water bath for extended periods of time.
    a. If media is more pink than peach, place media in the incubator and unscrew the cap, tilting it on top of the neck of the bottle to allow for gas exchange so the media pH returns to normal range 7.0 - 7.2. NSCs are sensitive to pH changes so only feed media that is peach in color indicating appropriate pH range.
3. On the day of feeding remove the plate from the incubator and transfer to the safety cabinet
4. Remove the old media from the cells and replace with 12ml fresh Neural StemCell Growth complete medium by slowly adding the media against the side of the dish then return the plate to the incubator. Do not add the media directly to the cell monolayer because the cells may come off the plate. Minimize the amount of time the cells are held out of the incubator.

IX. Dissociating hNSCs

1. hNSCs should be split for expansion every 3-5 days for optimal performance.
2. Prior to dissociating the cells prepare the laminin coated plates as described above.
    a. Each 100mm plate of hNSCs should yield enough cells to plate 4-6 x 100mm plates.
3. Working with multiple plates
    a. Only work with a stack of 4 x 100mm plates at a time

   b. Minimize time out of the incubator during the process
   c. Work swiftly but carefully to minimize cell loss
4. Remove old medium and replace with 2 ml trypsin
5. Take the plate of cells immediately to the microscope so that you can observe the trypsin action. Trypsinization of hNSCs takes less than a minute. Extended time in trypsin decreases viability.
6. Tap the dish against the palm of your hand to dislodge the cells and when the cells are free floating, return the plate to the safety cabinet
7. Aspirate 2.5ml Soybean Trypsin Inhibitor (STI) into a 5ml pipette, remove the lid and tilt the plate toward you then wash the cells down the dish followed by vigorously pipetting up and down 3-5 times.
8. Transfer the cell STI suspension into a 15ml conical tube.
9. Wash the plate with 2ml Neural StemCell Growth medium and add to the tube, pipetting up and down to ensure a homogenous cell suspension
10. Take an aliquot for cell counting
    a. Take 20ul cell suspension and add it to 20ul Trypan Blue. This provides a dilution factor of 2.
    b. Pipette up and down
    c. Take 10ul and add to the counting chamber of a hemacytometer
    d. Count the cells within the center grid
    e. Calculate the total cell count

Cell count X 10,000 X 2 X Volume = Total cell count

10,000 is a constant for the surface area of the counting chamber
2 is the dilution factor
Volume is the volume of the cell suspension being counted

    f. Determine the number of cells that can be plated for growth (refer to Table 1) or for differentiation (refer to Table 2)
11. Pellet the cells by centrifugation at 2000 RPM for 5 minutes.

X. Expanding NSCs

1. While the cells are in the centrifuge prepare the plates to receive the cells
   a. Add 10ml Neural StemCell Growth medium to each 100mm laminin coated plate and place in the incubator to allow the medium to equilibrate.
2. When the centrifuge stops remove the tube, take it to the biosafety cabinet and aspirate the supernatant taking care not to disturb the cell pellet.
3. Resuspend the cell pellet in enough Neural StemCell Growth complete medium to add 2ml cell suspension to each plate prepared in step X-1 above. Final volume/plate will be 12ml. Other plating formats can be used. Use Table 1 to calculate plating densities.
4. Rock plates back and forth, front to back then place into the incubator
5. Follow Feeding schedule in section VIII.

XI. Cryopreservation of NSCs

1. Follow dissociation methods in section IX.
2. After counting the cells divide the total cell count by 1.5×10^6 to determine the number of cryovials needed for freezing down the cells for long-term storage (assume freezing $1.5x10^6$ cells /ml/cryoviqal may freeze more cells/vial up to $1x10^7$/vial then adjust calculations accordingly).
3. Pellet the cells in all the tubes by centrifugation at 2000 RPM for 5 minutes.
4. While the cells are in the centrifuge print the labels needed for each cryovial
5. Place one label on each cryovial.
6. After the centrifuge stops, resuspend the cell pellet in enough Neural StemCell Freezing media to freeze 1.5×10^6 /ml
7. Transfer 1ml of neural freezing cell suspension into each cryovial

8. Transfer the cryovials into a controlled rate freezer. Alternatively vials can be placed into a styrofoam container that is then placed into a -80°C freezer overnight.
9. Next day transfer the vials of cells into the LN2 freezer for long-term storage.

XII. Planning for Differentiation of Human NPCs

1. Determine the number of cells you will need for your differentiation experiment (number of cells/multi-well plate x number of plates needed in the experiment – refer to Table 2) then follow the protocol for expansion of the hNSCs to achieve 80% of the total number of cells required for the differentiation experiment.
    a. The cells should be expanded in Neural StemCell Growth complete medium prior to pre-differentiation.
    b. For expansion plating densities refer to Table 1.
2. When you have expanded the NSCs to 80% of the cells needed for the differentiation experiment, the cells can be plated for pre-differentiation in Neural Transition complete medium. There will be additional growth of the NSCs while in Transition medium but not at the same rate as in Growth complete medium.
3. Following pre-differentiation, the cells will be dissociated and plated in Neural Differentiation complete medium in the multi-well plates required for the differentiation experiment (refer to plating densities in Table 2).

XIII. Plating the Cells for Pre-Differentiation

1. Follow the instructions for dissociation in section IX above.
2. Determine the number of laminin coated plates that can be plated with the cell **suspension (refer to Table 1 for plating densities for growth)**
3. While the cells are in the centrifuge prepare the plates to receive the cells
    a. For pre-differentiation the cells are plated on 100mm laminin coated plates in Neural Transition complete media.
4. Add 10ml Neural Transition medium to each 100mm laminin coated plate and place in the incubator to equilibrate
5. When the centrifuge stops remove the tube for the experiment, take it to the biosafety cabinet and aspirate the supernatant taking care not to disturb the cell pellet.
6. Resuspend the cell pellet in enough Neural Transition complete media to add 2ml cell suspension to the prepared plates required for pre-differentiation of the cells.
4. After adding the cells to the plates, carefully transfer the plates to the humidified incubator set at 6% CO_2, 2-5% O_2 and 37°C.
7. Pre-differentiation is carried out for 3 days.
8. Following pre-differentiation proceed to plating for differentiation.

XIV. Plating the Cells for Differentiation

1. Follow the instructions for dissociation in section IX above.
2. Determine the number of poly-d-lysine coated plates that can be plated with the cell suspension **(refer to table 2 for plating densities for differentiation)**.
3. While the cells are in the centrifuge prepare the plates to receive the cells.
4. When the centrifuge stops remove the tube for the experiment, take it to the biosafety cabinet and aspirate the supernatant taking care not to disturb the cell pellet.
5. Resuspend the cell pellet in enough Neural Differentiation complete medium to plate the multi-well plates.
    a. For multi-well plates use a multi-channel pipettor so the wells are plated evenly.
    b. For MEAs resuspend the cell pellet so that the final concentration is 60k cells per 5µl complete Differentiation medium and deposit each 5µl cell suspension drop directly on top of electrode area then gently place MEA in the incubator to allow cells to attach for approximately 1 hour then gently flood the chamber with media so as not to disturb the plated cells

6. Carefully transfer the plates to the humidified incubator set at 6% CO_2, 2-5% O_2 and 37°C.
7. On differentiation day 3 for the first feed, add an equal volume of Neural Differentiation complete media to each well.
8. Every third day thereafter, remove ½ of the media and replace with an equal volume of fresh Neural Differentiation complete media. The differentiation process is carried out for 28 days. Assays on mature neural cells can be conducted after 28 days in culture and for several weeks thereafter as long as the cells are fed every third day as described above.

Table 1. Growth plating density of human NSCs is calculated at ~20K cells/cm^2 for growth. The total number of NSCs plated per vessel are listed in this table. Use laminin coated plates for growth.

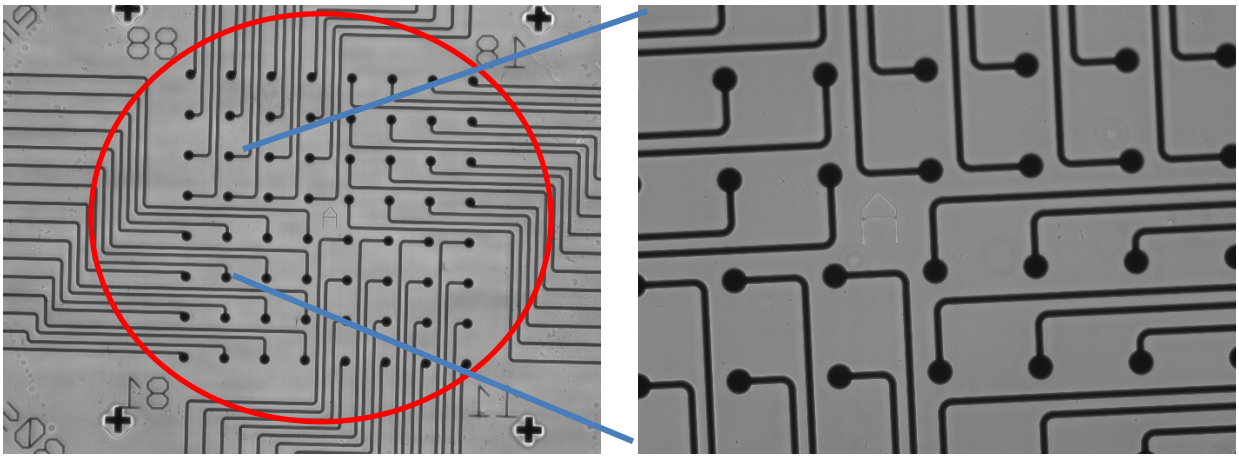
Dish/Flask Size	Growth Area (cm^2)	Working Volume (ml)	Maximum Volume (ml)	# NSCs/well	NSCs/Plate
100mm	58.1	12	15	1.20×10^6	1.2×10^6
60mm	21.3	8	10	4.30×10^5	4.3×10^5
35mm	9.6	3	5	1.92×10^5	1.92×10^5
6-Well	9.6	3	5	1.92×10^5	1.15×10^6
12-Well	3.8	2	4	7.60×10^4	9.12×10^5
4-Well	2.0	0.5	1	4.00×10^4	1.60×10^5
24-Well	2.0	1.0	2	4.00×10^4	9.60×10^5
48-Well	1.1	0.5	1	2.20×10^4	1.06×10^6
96-Well	.32	0.1	0.2	6.40×10^3	6.14×10^5
384-well	.086	0.05	0.1	1.80×10^3	6.60×10^5
2-Chamber	4.2	2	4	8.40×10^4	1.68×10^5
4-Chamber	1.8	1	2	3.60×10^4	1.44×10^5
8-Chamber	0.8	0.5	1	1.60×10^4	1.28×10^5
HyperFlask	1720	560	560	3.44×10^7	3.44×10^7

Table 2. Differentiation plating density of human NSCs is calculated at ~116K cells/cm^2 for differentiation on plates and dishes. The number of cells required per well or plate is listed in this table. Plating density for MEAs is much higher in order to measure activity in the neural network. Use poly-d-lysine or poly-d-lysine/laminin coated plates for differentiation.

Dish/MEA Size	Growth Area (cm^2)	Working Volume (ml)	Maximum Volume (ml)	NSCs /well	NSCs/Plate
100mm	58.1	10	5	6.74×10^6	6.74×10^6
60mm	21.3	6	3	2.47×10^6	2.47×10^6
35mm	9.6	2	1	1.11×10^6	1.11×10^6
6-Well	9.6	2	1	1.11×10^6	6.66×10^6
12-Well	3.8	1.5	0.75	4.41×10^5	5.29×10^6
4-Well	2.0	0.5	0.25	2.32×10^5	9.28×10^5
24-Well	2.0	1.0	0.5	2.32×10^5	5.57×10^6
48-Well	1.1	0.5	0.25	1.28×10^5	6.10×10^6
96-Well	.32	0.1	0.05	3.70×10^4	3.55×10^6
384-well	.086	0.05	0.025	1.00×10^4	3.84×10^6
2-Chamber	4.2	2	1	4.87×10^5	9.74×10^5
4-Chamber	1.8	1	0.5	2.10×10^5	8.40×10^5
8-Chamber	0.8	0.5	0.25	9.28×10^4	7.42×10^5
Single MEA	Electrode Area	0.5	1	6.00×10^4	6.00×10^4
12-Well MEA	Electrode Area	0.3	0.5	6.00×10^4	7.20×10^5
48-Well MEA	Electrode Area	0.1	0.2	6.00×10^4	2.88×10^6
96-Well MEA	Electrode Area	0.1	0.2	6.00×10^4	5.76×10^6

Figure 1. Light micrographs of Axion BioSystems' 12-well MEA

A. 12-well MEA surface 4x magnification. **B.** 12-Well MEA surface 10x magnification



A. Red Circle indicated the area where the 15µl drop of cells will be plated
B. Blue lines indicate enlarged area

NOTE: All the MEAs have the same surface area for plating the cells. The difference is in the number of electrodes on each MEA. 12-well MEAs have 64 electrodes, 48-well MEAs have 16 electrodes, and 96-well MEAs have 8 electrodes and all have 4 ground electrodes, one in each corner.

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From these organizations, a copy of the donor consent, medical history, and infectious disease testing for the presence of certain infectious diseases including HIV, HBV, HCV, CMV and RPR is received and reviewed to assure that the cells from each organ may be used for research purposes. Additionally, each tissue supplier working with PhoenixSongs has in place with the source facility material transfer agreements, as well as a review of the process by an internal or external ethics committee from each source facility. We review these documents at the initiation of any new donor source, and if there has been any change or revision to the process. These agreements as well as ethics reviews are reviewed and renewed periodically. Before isolating cells from these tissues, we review the donor consent paperwork to assure the isolation team that the donor, or donor's next of kin, has given consent for use of the cells obtained from these tissues for research purposes. The consent allows for the use and distribution of the cells both nationally and internationally without limitation. These reports and all other materials pertaining to the donor, the donated organ, and its processing are maintained in a physically secured location, with access available only to authorized persons. The records are stored in a manner to comply with US HIPAA regulations; the records are de-identified. Any reference to the donor's identity is removed.

