Toll Free: 866-702-0260
Phone: 203-433-4329
Fax: 203-208-0664
www.phoenixsongsbio.com

Methods for Differentiation of Human Dopaminergic Transitioned Neural Stem Cells (NSCs)

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
2. The dopaminergic transitioned 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 must 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: To obtain dopaminergic neurons it is essential to culture the dopaminergic transitioned NSCs in low oxygen conditions for optimal differentiation into a robust population of dopaminergic neurons. Atmospheric oxygen incubators may not be used for differentiation of dopaminergic transitioned NSCs.

II. Required Supplies

1. Dopaminergic Transitioned Neural Stem Cells (Cat. # 27001-009)
2. Dopaminergic Differentiation Medium (Cat. # 21002-250) includes the following components:
    a. Dopaminergic Differentiation Basal Medium
    b. Dopaminergic Differentiation Supplement Pack includes the following components:

Supplement Component
Dopa Differentiation Base Supplement (contains glutamine)
Laminin
BDNF
GDNF
Artemin
Neurtruin
Proprietary Factor 10

3. Poly-d-lysine coated plates (e.g. BD 96-well Biocoat 354640 or 384-well Biocoat 354836 or see coating method below)
4. Laminin/PDL coated plates (first coat with PDL then coat with Laminin -see method below)

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 Dopaminergic Differentiation Medium

1. Warm Dopaminergic Differentiation basal medium to room temperature
2. Thaw Dopa Differentiation Supplement and growth factors
3. Aseptically add supplements to basal medium in order
    a. Dopa Differentiation Base Supplement - transfer entire contents of the vial then rinse the vial with Dopa Differentiation basal medium
    b. Laminin and growth factors BDNF, GDNF, Artemin, Neurtruin and PF10 then rinse the vial with Dopa Differentiation 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 the complete media through 0.22µm filter unit
6. Store Dopaminergic Differentiation complete medium at 4°C in the dark until ready for use
7. Warm Dopaminergic Differentiation complete medium to room temperature immediately before use.
    a. If you prefer to warm in a water-bath, do not leave the Dopaminergic Differentiation complete medium in a water-bath for longer than 5 minutes as this may result in degradation of the proteins, growth factors and glutamine.
8. Use Dopaminergic Differentiation complete medium within two weeks. If you know you will not use the complete media withing two weeks, you may make smaller aliquots in bottles that can be sealed air tight, then store in the freezer.

V. Thawing Human DA-HIP9 Transitioned 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 PDL/laminin coated plate/MEA to receive recovered cells (see Table 1 for cell density and media volume).
4. Add volume of cold Dopa Differentiation complete medium needed to plate the cells to a conical tube.
5. 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.
6. 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.
7. 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
8. Douse the vial in alcohol dry and place inside the safety cabinet.
9. Open the vial and using a 5 ml pipette aspirate 2 ml of media from the tube into the pipette then gently remove the cell suspension from the vial with the pipette containing the media.
10. Transfer the cell suspension into the media in the tube. 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 tube
12. Pipette gently up and down too 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

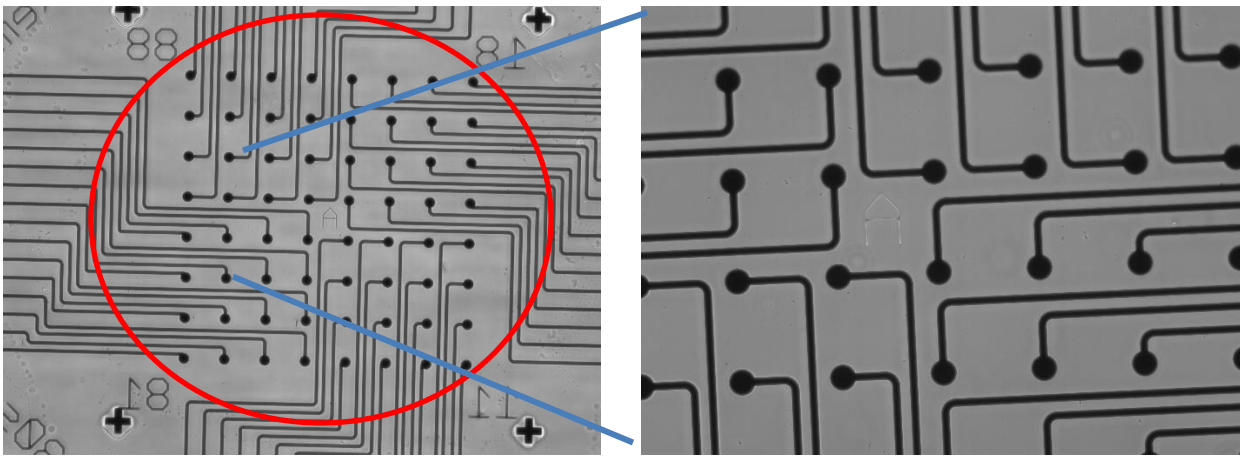
14. Calculate the total cell count for each the live cells and the dead cells
15. To determine percent live cells
    a. Total live cell count + total dead cell count = total cells
    b. Total Live cell count ÷ total cells x 100 = percent live cells
16. Transfer the cells into the plate format and density of your choice then put the plate 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 appropriate volume of Dopa Differentiation complete medium for your plate of choice (see Table 1 for cell density and media volume).

VI. Feeding Dopaminergic Transitioned Differentiating Cells

1. The first feed the day after recovery from cryopreservation is the only full feed. Subsequent feeds are half feeds to preserve the conditioned media.
2. The differentiating dopaminergic transitioned cells will need to be fed every 3-4 days. Remove ½ of the media and replace with an equal volume of fresh Dopa Differentiation complete media. The differentiation process is carried out for 28 days to achieve mature functional neural populations.
3. Assays on mature neural cells can be conducted after 28 days in culture. Dopaminergic neural populations can be used in multiple repeat assays beyond 72 days in culture as long and the cells undergo half feeds every 3-4 days as described above.

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.

Table 1. Differentiation plating density of human DA-Transitioned 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	15	6.74×10^6	6.74×10^6
60mm	21.3	6	10	2.47×10^6	2.47×10^6
35mm	9.6	2	5	1.11×10^6	1.11×10^6
6-Well	9.6	2	5	1.11×10^6	6.66×10^6
12-Well	3.8	1.5	4	4.41×10^5	5.29×10^6
4-Well	2.0	0.5	1	2.32×10^5	9.28×10^5
24-Well	2.0	1.0	2	2.32×10^5	5.57×10^6
48-Well	1.1	0.5	1	1.28×10^5	6.10×10^6
96-Well	.32	0.1	0.2	3.70×10^4	3.55×10^6
384-well	.086	0.05	0.1	1.00×10^4	3.84×10^6
2-Chamber	4.2	2	2	4.87×10^5	9.74×10^5
4-Chamber	1.8	1	2	2.10×10^5	8.40×10^5
8-Chamber	0.8	0.5	0.5	9.28×10^4	7.42×10^5
Single MEA	Electrode Area	0.5	0.5	6.00×10^4	6.00×10^4
12-Well MEA	Electrode Area	0.3	0.3	6.00×10^4	7.20×10^5
48-Well MEA	Electrode Area	0.1	0.1	6.00×10^4	2.88×10^6
96-Well MEA	Electrode Area	0.1	0.1	6.00×10^4	5.76×10^6

PhoenixSongs Ethics Policy on Human Cells and Tissues

Human stem cells isolated from fetal and neonatal tissues are distributed by PhoenixSongs for research purposes only. These donated tissues are obtained from several sources, including major university hospitals and US accredited organ procurement organizations. Consent is obtained from the donor, or donor's next of kin, and conforms to all country specific federal and local legal and ethical standards, including ICH, US HIPAA, US Uniform Anatomical Gift Act, US National Organ Transplant Act and UK HTA requirements.

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.

