

## Methods for Differentiation of Transitioned 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<sub>2</sub>
  - b. 2-4% O<sub>2</sub>
  - c. 95% humidity
  - d. 37°C

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

### II. Required Supplies

1. Transitioned Neural Stem Cells (Cat.# 27001-009)
2. 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

3. D-PBS with calcium and magnesium (Cat.# 41001-500)
4. Laminin coated plates (e.g BD Laminin Biocoat plates or see method below)
5. Poly-d-lysine coated plates (e.g. BD 96-well Biocoat 354640 or 384-well Biocoat 354836 or 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 4-8°C 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 4-8°C for up to three months.

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

<b>Dish/Flask Size</b>	<b>Growth Area (cm<sup>2</sup>)</b>	<b>Coating Volume</b>
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 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

### V. Thawing Human 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 Neural 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<sub>2</sub> 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

$$\text{Cell count} \times 10,000 \times 2 \times \text{Volume} = \text{Total cell count}$$

10,000 is a constant for the surface area of the counting chamber

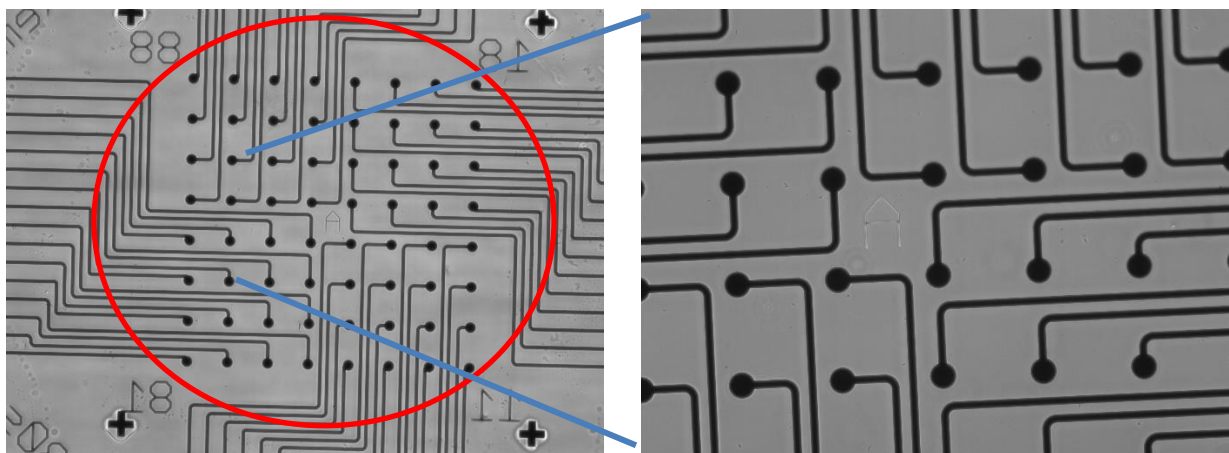
2 is the dilution factor

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 Neural Differentiation complete medium for your plate of choice (see Table 1 for cell density and media volume).

## VI. Feeding 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 pan-neural transitioned cells will need to be fed every 3-4 days. Remove ½ of the media and replace with an equal volume of fresh pan-neural 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. Pan-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 15 $\mu$ l 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.