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Methods for CryoNeurons™

PhoenixSongs' (PSB) human CryoNeurons™ are neurons that were produced from PSB NSCs that have been differentiated for 10-14 days then dissociated and cryopreserved in vials containing 5 million neurons. When thawed into PSB Neural Differentiation Media and plated on PDL or PDL/Laminin dishes, plates or MEAs the CryoNeurons™ continue the differentiation process and mature into neurons with functional synaptic activity by day 14-18 post plating. Our proprietary differentiation media formulation enables these neurons to mature and remain functional beyond 60 days *in vitro* in standard PDL/Laminin (PDL/L) coated dishes, plates and multi-electrode arrays (MEAs).

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as NuAire Class II Type A2 biosafety cabinet.
2. The CryoNeurons™ can be maintained with an antibiotic if required in order to prevent bacterial contamination. All our neurons have been tested with gentamycin (30μg/ml).
3. For best results, 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: For best results continue to culture CryoNeurons™ in low oxygen conditions for optimal differentiation and maturation into neurons. However, since CryoNeurons™ have already been committed to differentiation prior to cryopreservation an atmospheric oxygen incubator may be used with these neural cultures. It should be noted that this could result in lower percentage of neurons and high percentage of astrocytes and oligodendrocytes.

II. Required Supplies

1. Neural Differentiation Medium for pan-neural differentiation (Cat.# 21004-250) or Dopaminergic Differentiation Medium for dopaminergic differentiation (Cat.# 21002-250)

2. Poly-d-lysine /Laminin coated plates (e.g. BD 96-well Biocoat 354596 or see coating method below)

III. Coating Dishes, Plates & MEAs with Poly-D-Lysine/Laminin

Coating with Poly-D-Lysine (PDL)

1. Make PDL (Sigma P7280) solution at 50ug/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 PDL solution per cell culture vessel and let set in hood for at least 5 minutes.
3. Remove PDL 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 PDL plates with Laminin

1. Make laminin (Sigma L-2020) solution at 10ug/ml in a balanced salt solution for dishes and plates or 20ug/ml for MEAs 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 or MEA, add appropriate volume of laminin solution (refer to Table 1) and let set in hood for 2 hours.
3. Remove laminin and use immediately or placed in sterile bag and store at 4-8ºC for up to three months.

Table 1. Tissue Culture Dishes, Plates and MEAs Volume for Coating Plates

Dish/Plate/MEA Size	Growth Area (cm^2)	Coating Volume
100mm	58.1	5 ml
60mm	21.3	3 ml
35mm	9.6	1.5 ml
6-Well	9.6	1.5 ml
12-Well	3.8	300 µl
4-Well	2.0	200 µl
24-Well	2.0	200 µl
48-Well	1.1	100 µl
96-Well	.32	50 µl
384-Well	.136	30 µl
2-Chamber	4.2	300 µl
4-Chamber	1.8	200 µl
8-Chamber	0.8	100 µl
Single MEA	Electrode Area	80 µl
12-Well MEA	Electrode Area	80 µl
48-Well MEA	Electrode Area	80 µl
96-Well MEA	Electrode Area	80 µl

IV. 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

V. Preparing Neural or Dopaminergic Differentiation Media

1. Follow the instructions that accompany the Differentiation Media for making the media.
2. Use Neural or Dopaminergic Differentiation complete medium within two weeks

VI. Thawing Human CryoNeurons™

1. Warm the water-bath to 37°C.
2. Warm Neural or Dopaminergic Differentiation complete media to room temperature or in the water-bath for a maximum of 5 minutes.
3. Remove the media from the water-bath, douse with 70% alcohol, dry and place inside the biosafety cabinet.
4. Using a 10ml pipet, transfer 5ml media into a 15ml conical tube labeled with the cell ID and set aside.
5. To thaw the CryoNeurons™, 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 70% alcohol dry and place inside the safety cabinet.
9. Open the vial then using a 1-2ml pipette gently remove the cell suspension from the vial.
10. Transfer the cell suspension into the media already in the 15ml 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. Gently pipet up and down to ensure a homogeneous cell suspension and an accurate cell count.
12. Immediately following pipetting 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. Let the tube of cells remain in the hood for at least another 15 minutes before centrifugation to allow the cell membranes to recover after thawing.
15. Calculate the total cell count for each the live cells and the dead cells
16. 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

17. Determine plating cell number and media volume (use Table 2 as a guide) for the CryoNeurons™ cell suspension. Optimal plating density for each assay may vary so we recommend doing a plating density study first to determine the optimal density for your studies.
18. Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature.
19. Remove supernatant and resuspend cell pellet in the volume of media determined in step 17 above for plating the CryoNeurons™ and gently pipet up and down to produce a homogeneous cell suspension.
20. Transfer cell suspension into the cell culture plates, dishes or MEAs.
21. Carefully transfer the plates, dishes or MEAs to the humidified incubator set at 6% CO_2, 2-5% O_2 and 37°C.

VII. Feeding CryoNeurons™

1. CryoNeurons™ should be fed twice per week. Typically feed on Monday and Friday to avoid weekend feeding.
2. Warm Neural or Dopaminergic Differentiation 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 in the bottle is more pink than peach, place bottle 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. CryoNeurons™ 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. For the first feed after thawing the cells simply add an equal volume of fresh Neural Differentiation complete media 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. Minimize the amount of time the cells are held out of the incubator. .

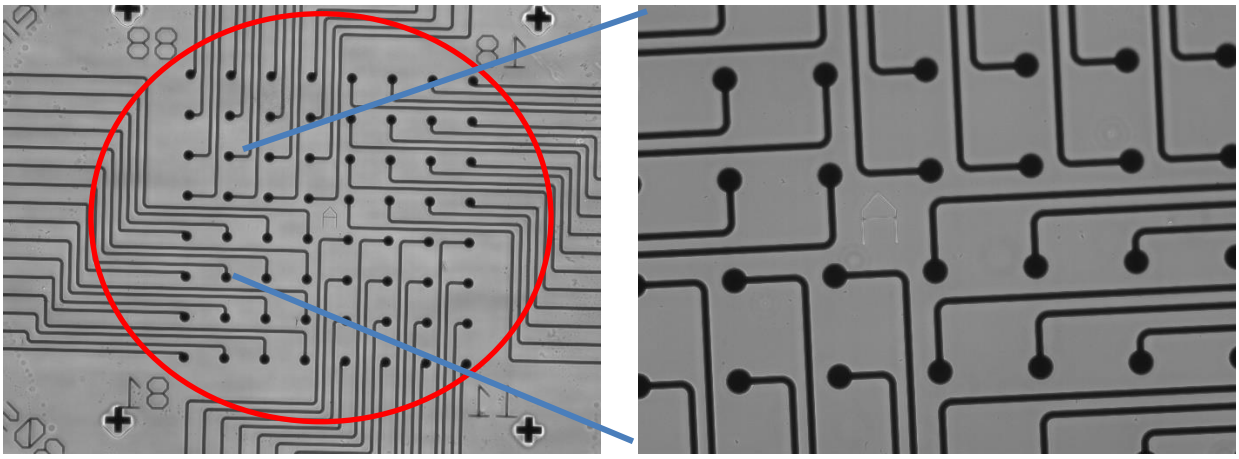
5. For subsequent feedings, remove ½ of the old media from the cells and replace with an equal volume of fresh Neural or Dopaminergic Differentiation complete medium by slowly adding the media against the side of the dish then return the plate to the incubator. Do not add the media directly to the cell monolayer because the cells may come off the plate. Minimize the amount of time the cells are held out of the incubator.
6. For CryoNeurons™ frozen on day 10 of differentiation continue differentiation for another 18 days to ensure mature functional synapses. For CryoNeurons™™ frozen on day 14 of differentiation continue differentiation for another 14 days.
7. CryoNeurons™ may be kept in culture for greater than 60 days if regular feeding is maintained. Neural Differentiation Media will support maintenance and survival of mature neural cell populations.

Table 2. Plating Density for CryoNeurons™ is calculated at ~165K cells/cm^2. 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. See Figure 1 for MEA description and photos. Use PDL or PDL/Laminin coated plates.

Dish/MEA Size	Growth Area (cm^2)	Plating Volume (ml)	Feeding Volume (ml)	CryoNeurons™ /well	CryoNeurons™/Plate
100mm	58.1	10	5	9.59×10^6	9.59×10^6
60mm	21.3	6	3	3.51×10^6	3.51×10^6
35mm	9.6	2	1	1.58×10^6	1.58×10^6
6-Well	9.6	2	1	1.58×10^6	9.50×10^6
12-Well	3.8	1.5	0.75	6.27×10^5	7.52×10^6
4-Well	2.0	0.5	0.25	3.30×10^5	1.32×10^6
24-Well	2.0	1.0	0.5	3.30×10^5	7.92×10^6
48-Well	1.1	0.5	0.25	1.82×10^5	8.71×10^6
96-Well	.32	0.1	0.05	5.28×10^4	5.07×10^6
384-well	.086	0.05	0.025	1.42×10^4	5.45×10^6
2-Chamber	4.2	2	1	6.93×10^5	1.39×10^6
4-Chamber	1.8	1	0.5	2.97×10^5	1.19×10^6
8-Chamber	0.8	0.5	0.25	1.32×10^5	1.06×10^6
Single MEA	Electrode Area	1.5	2	8.50×10^4	8.50×10^4
12-Well MEA	Electrode Area	1.5	2	8.50×10^4	1.02×10^6
48-Well MEA	Electrode Area	0.5	1.0	8.50×10^4	4.08×10^6
96-Well MEA	Electrode Area	0.1	0.2	8.50×10^4	8.16×10^6

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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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, US accredited organ procurement organizations or obtained from Advanced Bioscience Resources, Inc. 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.

