Standard Operating Procedure

Derivation of Single Neural Rosette Tissues with RosetteArray™ Plates

Overview: This document provides step-by-step guidance for using RosetteArray plates to derive micropatterned neural rosette tissues. The default culture format utilizes 96-well plates and changes are noted where applicable for different well plate sizes.

Equipment Required:

- Cell culture incubator
- Biosafety cabinet
    - Vacuum aspirator
- Brightfield microscope
- Cell counter or hemacytometer
- 4°C refrigerator
- Liquid nitrogen storage container
- Benchtop pipettor
- Micropipettes
    - 1000 µL
    - 200 µL
    - 20 µL
    - 200 µL multi-channel
- Centrifuge tube racks

Materials Required:

NOTE: All of the following listed items must be serologically sealed or autoclave sterilized prior to use.

- 10 mL pipettes
- 5 mL pipettes
- 15 mL conical centrifuge tubes
- 50 mL conical centrifuge tubes
- 1.5 mL microcentrifuge tubes
- 1000 µL micropipette tips
- 200 µL micropipette tips
- 20 µL micropipette tips
- 25 mL multi-channel pipette media reservoirs
- Trypan blue (alternative live/dead indicator)
- Pasteur pipettes
- Cell culture-grade dimethyl sulfoxide (DMSO)
- Matrigel® Matrix, Corning® (or alternative extracellular matrix [ECM] for cell adherence)
- 4% paraformaldehyde in phosphate-buffered saline (PBS)

- Antibodies for N-cadherin and Pax6
    - Species-corresponding secondary antibodies
- ProLong™ Glass Antifade Mountant, Invitrogen™ (P36980)
- Donkey serum
- Triton-X 100

NOTE: If you purchased the RosetteArray plates alone, you will need to supply your own human pluripotent stem cell (hPSC) maintenance and differentiation mediums. We recommend the following formulations:

- Essential 8™ Medium, Gibco (A1517001)
- Essential 6™ Medium, Gibco (A1516401)

NOTE: Commercial media contains 10X the concentration of insulin and may alter experimental timelines.

- Y-27632 ROCK inhibitor
- Penicillin/Streptomycin (10,000 u/mL)
- DMEM/F-12

Protocol Overview:

1. Prepare RosetteArray plates for cell culture
2. Seed hPSCs on RosetteArray plates
3. Differentiate hPSCs with RosetteArray technology
4. Immunostain rosette tissues on RosetteArray plates
5. Image rosette tissues on RosetteArray plates

Protocol:

1. Prepare RosetteArray plates for cell culture (Day -2)

RosetteArray plates are assembled in a sterile environment. To ensure maintenance of sterility, we recommend rinsing plates 3 times with sterile, cell culture-grade PBS in a sterile biosafety cabinet. Additionally, we recommend culturing cells in antibiotic-containing media.

NOTE: Do not remove the RosetteArray plates from the vacuum sealed packaging until you are ready to begin culture.

NOTE: All steps should be performed in a sterile biosafety cabinet.

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1. Cut open one side of the vacuum sealed packaging and remove the RosetteArray plate.

2. In a biosafety cabinet, rinse every well of the 96-well plate with 200 µL sterile, cell culture-grade PBS **3 times**.
3. Resuspend Matrigel to a final concentration of 0.083 mg/mL in DMEM/F-12 (or alternative base media) containing Penicillin/Streptomycin (Pen-Strep).
4. Pipette 100 µL Matrigel mixture to each well of the RosetteArray plate.

NOTE: Due to media evaporation, it is not recommended to culture cells in the wells along the exterior of the plate. Instead, culture in the interior 60 wells of a 96-well plate.

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5. Place the RosetteArray™ plate in a 37°C cell culture incubator for at least 3 hours to allow matrix adsorption. Overnight incubations are standard practice, but we do not recommend exceeding 24 hrs.

2. Seed hPSCs on RosetteArray plates (Day -1)

RosetteArray plates are compatible with all hPSC lines, both cryo-preserved and fresh from sub-culture. As rosette formation is an artifact of neural differentiation, it is theoretically possible to derive rosettes on RosetteArray plates using a variety of differentiation protocols. However, we recommend adhering to the following seeding and differentiation protocol, particularly the media compositions.

NOTE: All steps should be performed in a sterile biosafety cabinet.

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1. Prepare 35 mL E8/Pen-Strep media containing 10 µM ROCK inhibitor (ROCKi) using a 50 mL conical centrifuge tube.
2. Remove cryo-preserved hPSCs from liquid nitrogen storage and thaw in a 37°C bath.
3. Once thawed, place the hPSC vial in the biosafety cabinet. Use a wide bore pipette tip (or a 5 mL pipette) to mix cells once, then remove the cell mixture from the vial and pipette into a fresh 15 mL centrifuge tube.
4. In dropwise fashion, pipette 4 mL E8/Pen-Strep/ROCKi media into the 15 mL tube to slowly dilute the cryo-preservation media.
5. Gently invert the tube 2 times to ensure proper mixing.
6. Centrifuge the cell suspension for 3 mins at 200xg.
7. Aspirate the media being careful not to disturb the cell pellet.
8. Add 6 mL E8/Pen-Strep/ROCK media, pipetting gently.
9. Using a P1000 wide bore pipette tip, resuspend the cell pellet in the added media.

10. Count cell density with your preferred method.

> **Example:** You have a resuspended cell concentration of $1.5x10^6$ cells/mL. You want to seed the interior 60 wells of a 96-well RosetteArray plate at the ideal seeding density of $2x10^5$ cells/cm^2. Ideal seeding density does not differ between well plate layouts.
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> - 200 µL/well X 60 wells X *1.25 media excess* = 15 mL total media
> - $2x10^5$ cells/cm^2 X 0.32 cm^2/well X 60 wells X *1.25 excess* = $4.8x10^6$ cells required
> - $4.8x10^6$ cells required / $1.5x10^6$ cells/mL = 3.2 mL cell suspension required
> - 15 mL total media – 3.2 mL cell suspension = 11.8 mL additional media

11. Resuspend cells in E8/Pen-Strep/ROCKi media to ensure a $2x10^5$ cells/cm^2 seeding density.
12. Pipette or decant the entire media mixture into a 25 ml multi-channel media reservoir.
13. Using a multi-channel 200 µL micropipette, remove 90 µL of the Matrigel solution from the RosetteArray plate.
14. Transfer 200 µL of the cell suspension into the desired wells of the 96-well RosetteArray plate.
    a. Gently pipette up and down once to resuspend the cell mixture before each transfer.
    b. We recommend seeding by rows (i.e., Row B to row G) vs. by columns (i.e., column 2 to column 11).
15. When all desired wells have been seeded, pipette 200 µL sterile, cell culture-grade PBS into the peripheral wells as an evaporation buffer.
16. Allow the plate to sit in the Biosafety Cabinet with the lid on for 10 minutes to ensure proper seeding.
    a. DO NOT shake the plate in an attempt to evenly disperse the cells.
17. Gently place the seeded RosetteArray plate in a 37°C incubator.
18. Allow at least 3 hours for cells to adhere before removing the plate from the incubator.

3. Differentiate hPSCs with RosetteArray Technology (Day 0-7)

The differentiation timeline for generation of hPSC-derived neural rosette tissues is 8 days, not including the day of seeding (Day -1). Day 0 is considered the day hPSC maintenance media (E8) is replaced with differentiation media (E6). In the 96-well format, a 75% media change is performed on Day 0 and 50% media changes are performed every day thereafter. For all other plate formats (6-well & 24-well) 100% media changes are performed on Day 0 and 50% media changes are performed every day thereafter. We recommend differentiation media containing 0.1% DMSO by volume to enhance neural differentiation and neural rosette formation efficiency. When performing developmental neurotoxicity screening with RosetteArrays it is critical to maintain 0.1% DMSO concentration across experimental conditions to ensure no solvent-related variability. To ensure uniformity of DMSO concentration and experimental treatment

concentration while performing daily 50% media changes, we recommend doubling the concentration of DMSO and treatment compound for Days 1-6.

NOTE: All steps should be performed in a sterile biosafety cabinet.

Day 0

1. Remove the RosetteArray plate from the incubator. Check seeding efficiency and ensure proper restriction of cell seeding to micropatterned regions.

NOTE: Media change should not be performed earlier than 16 hours after seeding.

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2. Prepare 190 µL of E6 media containing Penicillin/Streptomycin and 0.1% DMSO for every well of a 96-well plate in culture.
    a. 6-well format: 5 mL/well
    b. 24-well format: 1.5 mL/well
3. Using a multi-channel micropipette, remove 150 µL E8/Pen-Strep/ROCK media per well and transfer it to a waste media reservoir.
    a. 6-well format: 4 mL/well
    b. 24-well format: 1 mL/well
4. Pipette fresh E6/Pen-Strep/0.1%DMSO media to a multi-channel media reservoir.
5. Using a multi-channel micropipette, transfer 150 µL E6/Pen-Strep/0.1% DMSO for every well in culture.
    a. 6-well format: 4 mL/well
    b. 24-well format: 1 mL/well

NOTE: If performing developmental neurotoxicity screening, replace E8/Pen-Strep/ROCK media with E6/Pen-Strep/0.1% DMSO media containing your compounds of interest.

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6. Place the RosetteArray plate back in the incubator.

Days 1-6

NOTE: It is recommended to refresh all evaporation buffer wells with 200 µL PBS on Day 3.

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1. Remove the RosetteArray plate from the incubator. Check cell viability and ensure proper restriction of cell seeding to micropatterned regions.
2. Prepare 125 µL of E6/Pen-Strep/0.2% DMSO for every well of a 96-well plate in culture.
    a. 6-well format: 2.5 mL/well

   b. 24-well format: 0.75 mL/well
3. Using a multi-channel micropipette, remove 100 µL spent E6/Pen-Strep/0.1% DMSO media per well and transfer it to a waste media reservoir.
   a. 6-well format: 2 mL/well
   b. 24-well format: 0.5 mL/well
4. Pipette fresh E6/Pen-Strep/0.2% DMSO media to a multi-channel media reservoir.
5. Using a multi-channel micropipette, transfer 100 µL E6/Pen-Strep/0.2% DMSO media into every cultured well.
   a. 6-well format: 2 mL/well
   b. 24-well format:0.5 mL/well

NOTE: When performing developmental neurotoxicity screening with RosetteArrays, double the concentration of your tested compound for each experimental condition as well.

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6. Place the RosetteArray plate back in the incubator.

Day 7

Seven days is typically sufficient to observe neural differentiation and rosette formation in hPSC lines. Extended culture is possible, but tissues will continue to grow and could off of the substrate surface. As such, we recommend either fixing and staining tissues on Day 7, or embedding the tissues in a hydrogel for continued culture. Here, we detail a fixation protocol compatible with RosetteArray plates.

1. Remove the RosetteArray plate from the incubator.
2. Carefully remove all the media from each well and place it in a waste reservoir.
3. Rinse each well with a sterile, cell culture-grade PBS, i.e. (100 µL PBS of a 96-well plate).
   a. 6-well format: rinse with 2 mL/well
   b. 24-well format: rinse with 0.5 mL/well
4. Pipette an equivalent volume of cold 4% paraformaldehyde in PBS onto each well and allow fixation to occur for 10 mins. Do not exceed 15 mins of fixation.
5. Removed all paraformaldehyde and place it in a waste reservoir.
6. Rinse each well cultured with an equivalent volume of sterile, cell culture-grade PBS twice.
7. Add the volume of PBS per well used to rinse and store the fixed plate at 4°C for up to a week.
   a. It is not recommended to store fixed tissues on RosetteArray plates for more than a week 4°C.

4. Immunostain rosette tissues on RosetteArray plates

To analyze the microarrayed tissues for cell viability, neural differentiation, singular neural rosette emergence efficiency, and neural rosette morphology, the tissues must be stained with a nuclear stain (typically DAPI) and antibodies for Pax6 and N-cadherin. This can be

accomplished using a variety of protocols. Our standard approach will be outlined briefly here. We use donkey serum to block non-specific binding and Triton-X 100 to permeabilize cell membranes.

1. Prepare 1 mL PBS with 5.0% donkey serum and 0.3% Triton-X 100 (PBS-DT) for every well of a 96-well plate to be stained.
2. Manually remove PBS rinse from culture wells of RosetteArray plate(s).
3. Using a multi-channel pipette, transfer 100 µL of PBS-DT for every well to be stained.
    a. Allow blocking with PBS-DT for at least 1 hour at RT or overnight at 4°C.
4. Resuspend Pax6 and N-cadherin antibodies in 110 µL PBS-DT for every well of the 96-well plate to be stained.
    a. Optimal antibody dilution should be determined *a priori*.
5. Using a multi-channel pipette, transfer 100 µL PBS-DT containing N-cadherin and Pax6 antibodies.
    a. Allow to stain overnight at 4°C.
6. Using a multi-channel pipette, remove 100 µL PBS-DT/N-cadherin/Pax6 antibody mixture and rinse 3 times with 100 µL PBD-DT.
    a. You may use an orbital shaker on gentle settings but this is not necessary.
7. Resuspend secondary antibodies with DAPI in 110 µL PBS-DT for every well of the 96-well plate to be stained.
8. Using a multi-channel pipette, remove 100 µL PBS-DT and replace with 100 µL PBS-DT with secondary antibodies and nuclear stain.
    a. Allow to stain at RT for at least 2 hours.
    b. You may use an orbital shaker on gentle settings but this is not necessary.
9. Using a multi-channel pipette, remove 100 µL PBS-DT with secondary antibodies and the nucleic acid stain.
10. Rinse each well with 100 µL PBS-DT twice and with 100 µL PBS once.
    a. You may use an orbital shaker on gentle settings but this is not necessary.
11. Confirm success of staining using a fluorescent microscope.
12. Remove the remaining PBS and seal each stained well of a 96-well plate with approximately 70 µL of ProLong Glass Antifade Mountant.
    a. Allow this to cure overnight at RT before performing any imaging.

5. Image rosette tissues on RosetteArray plates

Rosette tissues can become up to ~40 µm thick. Therefore, we recommend acquiring images of multiple tissue sections through confocal microscopy to reliably capture rosette morphology of an individual tissue. For each well of a 96-well plate, select 40 tissues at random an obtain at least 5 image slices/Z-planes of each tissue. In order to ensure the entire micropatterned region is captured in a single field of view, image using a 20X objective and 2.5X magnification factor.