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Protocol

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Generation of 'semi-guided' cortical organoids with complex neural oscillations

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SUPPLEMENTARY METHODS

AggreWell forced aggregation initiation

Unique materials: AggreWell™ Anti-Adherence Rinsing Solution (STEMCELL Technologies, Cat #07010), AggreWell™800 24-well Plate (STEMCELL Technologies, Cat #34811)

Day 0: Prepare Aggrewell-800 plate (Timing: 20 minutes)

1. Warm 75 mL of basal DMEM/F12.
2. Sterilize with 70% ethanol and open the AggreWell plate in a biosafety cabinet. Do not expose the plate itself to ethanol or isopropanol solvents.
3. Add Anti-Adherence Rinsing Solution to the plate, using 500 µL for each well of a 24-well plate.
4. Using a swing bucket rotor centrifuge with plate holders, centrifuge the AggreWell plate with appropriate counterbalance for 5 minutes at 1300g.
5. After centrifugation, check the plate under an inverted microscope to confirm that there are no bubbles in the microwells. The Anti-Adherence Rinsing Solution needs to coat all surfaces of the microwells for proper use. If bubbles are present in microwells, centrifuge again for 5 minutes at 1300g.
6. After centrifugation, aspirate the Anti-Adherence Rinsing Solution and rinse each well with warm basal DMEM/F12, by adding 2 mL of basal media to each well of the 24-well plate.
7. Aspirate the rinse and add 1 mL of warm basal DMEM/F12 to each well of the 24-well plate. Store the plate in a 37°C humidified incubator until use. AggreWell plates with unused wells can be stored in a 37°C humidified incubator for 1 month, as long as they do not dry out.
8. Prepare an equivalent counterbalanced 24-well plate with water, with 1 mL per well of a 24-well plate. Set aside.

Day 0: Dissociation of iPSCs (Timing: 25 minutes) and plating to AggreWell plate (Timing: 15 minutes) for the generation of 3D embryoid bodies (EBs) via forced aggregation

9. Grow cells to about 85 percent confluence, and ensure that they are free of spontaneous differentiation before being passaged.
10. Briefly warm mTeSR 1 and Accutase in a 37°C water bath. Supplement pre-warmed mTeSR 1 with 10µM Y-27632 ROCK inhibitor, 10µM SB, and 1µM Dorso (mTeSR 1 neural induction media) when ready to be used. ! CRITICAL STEP For all supplement additions throughout differentiation, add supplements after base media has been warmed.
11. Gently dissociate iPSCs by aspirating cell culture media from the iPSC dish and adding 2mL of warm Accutase per 6 cm dish, for 5 to 7 minutes. Check for cell detachment starting at 4 minutes. Once most of the cells are detaching, which can be seen as cells sliding across the dish with a quick dish tilt, add 4mL of media to the plate to dilute the

Accutase.

12. Using a serological pipet, collect the cell suspension and pass the suspension through a 40µm cell strainer placed on top of a 50mL conical tube to remove undissociated clumps. Hold the serological pipet vertically, not at an angle, and gently dispense the cell suspension against the filter.
13. Centrifuge the cell suspension for 5 minutes at 100g.
14. Aspirate the supernatant and tap gently to loosen the pellet. Resuspend in 2 mL of mTeSR 1 neural induction media with ROCKi. If processing multiple plates for dissociation and replating, resuspend cells in an appropriate higher volume of media (e.g. for three 6 cm plates, resuspend in 6mL media).
15. Take a cell count using Trypan Blue exclusion. Do not proceed if cell viability is below 80%. Ideally, cell viability should be greater than 90% to start the organoid protocol.
16. Using the viable cell count, create a cell suspension of 3 million cells per mL.
17. Retrieve the prepared AggreWell plate, aspirate the basal media, and add 1 mL of cell suspension per well. Unused wells can remain in basal media, but be sure the plate contains 1 mL per well in every well prior to centrifugation. ! CRITICAL STEP Optimal starting seeding density may vary between iPSC lines, particularly in certain disease genotype iPSC lines. Determine optimal starting seeding density empirically, usually between 2 to 3 million cells per well of an AggreWell 24-well plate.
18. Centrifuge the AggreWell plate with appropriate counterbalance (from step 8) for 3 minutes at 100g to draw down the cell suspension evenly into the microwells.
19. Gently add an additional 1 mL of mTeSR 1 neural induction media to each well, for a final volume of 2 mL per well.
20. Check the plate under an inverted microscope to confirm that cells are evenly distributed into the microwells.
21. Place the AggreWell plate on a completely level incubator shelf in a 37°C incubator with 5% CO_2 and 95% humidity for 24 hours.

Day 1: Retrieve EBs from AggreWell plate (Timing: 30 minutes) and begin neural induction

22. Prepare mTeSR 1 neural induction media by warming mTeSR 1, then supplementing with 10µM SB, and 1µM Dorso.
23. Sterilize scissors. Spray with 70% ethanol, wipe down, and re-spray with 70% ethanol. Allow to air dry in a biosafety cabinet.
24. Retrieve the AggreWell plate and observe under an inverted microscope to confirm the formation of even sized EBs in each microwell. ! CRITICAL STEP Do not continue if EBs did not form or are markedly different in size due to uneven aggregation. If plating and comparing control and disease genotype iPSC lines, both lines should have formed similarly sized EBs when plated in the same AggreWell plate. If iPSCs incorporated into EBs at markedly different efficiency, leading to distinctly differently sized EBs between control and disease iPSC lines, investigate instead of proceeding. Forced aggregation should ideally produce EBs of consistent size between cell lines, assuming high quality high viability starting material.

25. Place a 40µm cell strainer on top of a 50 mL conical tube. Each well of an AggreWell plate will be collected and transferred to 1 well of a 6 well plate, sequentially. The same 40 µm cell strainer can be re-used for the same cell line, but additional cell strainers may be needed if many wells are being processed and the cell strainer clogs.
26. Use sterilized scissors to cut the tip off a p1000 tip to create an extra-large bore tip. Alternatively, use a 5 mL serological pipet. Draw up half the culture media (1 mL) from one well of the AggreWell plate, and dispense firmly back into the well to dislodge EBs. Draw up again quickly, to collect EBs in suspension and transfer to the 40µm cell strainer.
27. Use 1 mL of media to rinse the well in the same manner to dislodge and collect any remaining EBs, and transfer to the 40µm cell strainer, rinsing the strainer during the dispense. Confirm successful retrieval of EBs using an inverted microscope, or repeat the rinse step if necessary.
28. Carefully invert the strainer containing the retrieved EBs over 1 well of a 6 well plate.
29. Wash the underside of the strainer with 3 mL of mTeSR 1 neural induction media to dislodge the EBs from the strainer into the 6 well plate.
30. Repeat for each well of the AggreWell plate that was used.
31. Confirm successful retrieval, even sized EBs, and approximately even number of EBs per well using an inverted microscope and visual examination of the 6 well plate.
32. Place the 6 well plate on the orbital shaker, set to 95 rpm, and continue culture per the organoid protocol from Day 2 onwards (steps 13 through 20).