Title	Differentiating EBs (Rosette Formation) and Midbrain Specification
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Adapted from -	Gibco Protocol
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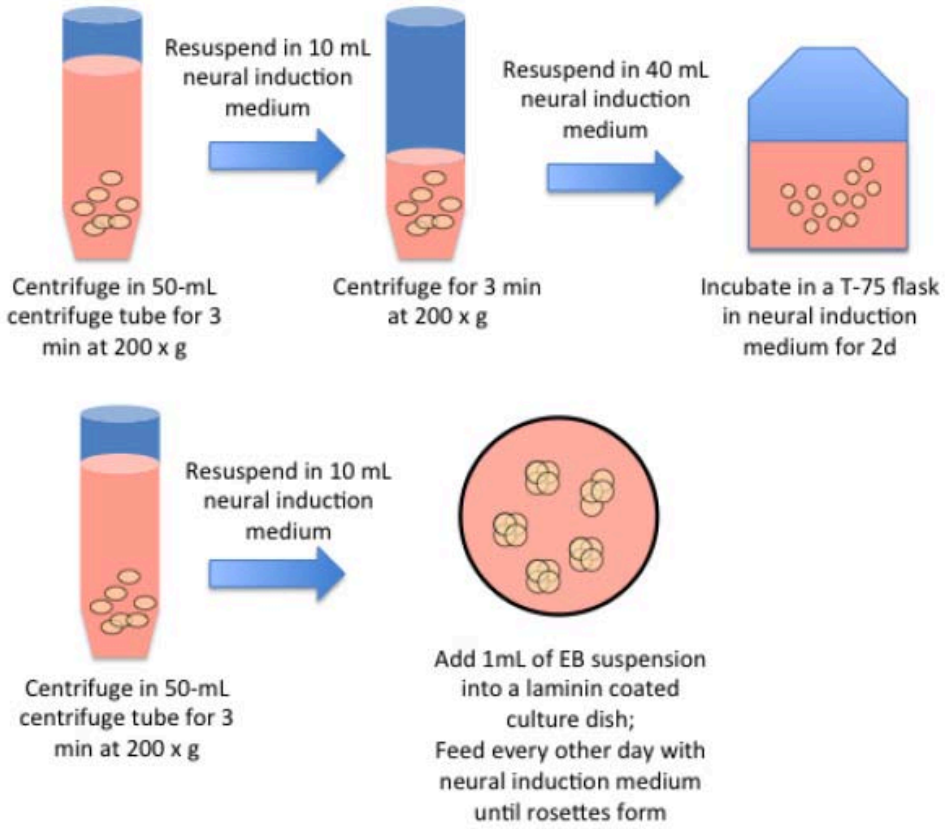
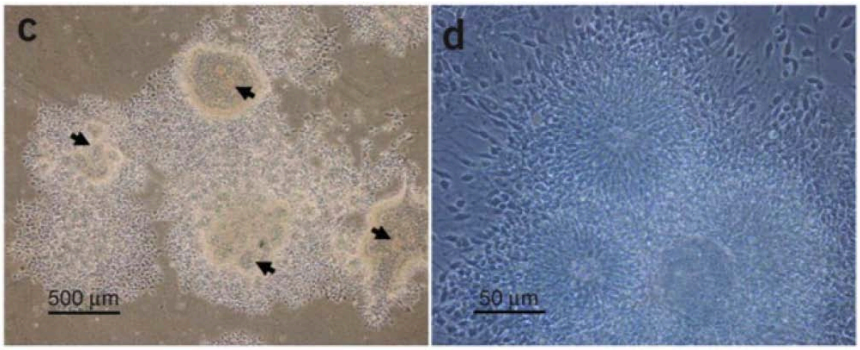
❖ Introduction:





Neural rosettes formed 2 days after replating (c) and magnified image of neural rosettes (d)[1]

❖ Protocol:

1. After culturing the EBs in EB medium for 4 days, transfer the EBs from one T- 75 flask into a 50- mL centrifuge tube and centrifuge for 3 minutes at 200 × g.
2. Aspirate the EB medium and resuspend the EBs in 10 mL of pre- warmed neural induction medium.
3. Centrifuge the EBs for 3 minutes at 200 × g.
4. Aspirate the supernatant and resuspend the EBs in 40 mL of pre- warmed neural induction medium. Transfer the EBs into a fresh T- 75 flask and incubate the EBs in neural induction medium for 2 days in a 37 C incubator with a humidified atmosphere of 5% CO2. After the EBs float in the neural induction medium for 2 days, they are ready to be differentiated.
   Note: If the EB attach to the flask, use a 5- mL pipette to blow the attached EBs off the bottom of the flask.
5. Dilute laminin in D- PBS to 20 ug/mL and coat ten 100- mm culture dishes using 2.5-3 mL of laminin for each dish. Incubate the laminin- coated culture dishes in a 37 C incubator for several hours.
6. After incubation, aspirate the laminin and add 10 mL of pre- warmed neural induction medium into each 100 mm dish.
7. Transfer the EBs from the T- 75 flask into a 50- mL tube and centrifuge for 3 minutes at 200 × g.
8. Aspirate the supernatant and resuspend the EBs in 10 mL of pre- warmed neural induction medium.
9. Gently shake the 50- mL tube containing EBs to distribute the EBs evenly and add 1 mL of EB suspension into each laminin- coated culture dish.
10. Move the culture dishes in several quick back- and- forth and side- to- side motions to disperse the EBs across the surface of the dishes. Place the dishes gently in a 37 C incubator with a humidified atmosphere of 5% CO2.
11. Feed the EBs every other day with fresh pre- warmed neural induction medium until early rosettes form (approximately 2-3 days).
12. To direct the neural precursors to the midbrain fate, feed the differentiating EBs every other day with neural induction medium containing 100 ng/mL FGF- 8b and 200 ng/mL sonic hedgehog (SHH) for 5-6 days.
    Note: Plate the EBs at a density of 200-250 per one 100- mm dish. Generally, all EBs from hESCs cultured in one 100- mm dish can be plated into eight to ten 100- mm dishes. The variation is from the confluence of hESCs and efficacy of EB formation

❖ Materials:

EB medium
neural induction medium
laminin
D-PBS

Neural Induction Medium	
Component	Amount
D-MEM/F-12	98 mL
N-2 Supplement	1 mL
NEAA	1 mL
Basic FGF Solution	200 µL
Heparin Solution	100 µL

- Troubleshooting:

References:

1. Myung-Soo Cho, Dong-Youn Hwang & Dong-Wook Kim Efficient derivation of functional dopaminergic neurons from human embryonic stem cells on a large scale. Nature Protocols 3, 1888 - 1894 (2008).