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iPSC NGN2-BRN3A Differentiation Protocol	Revision Number: 01
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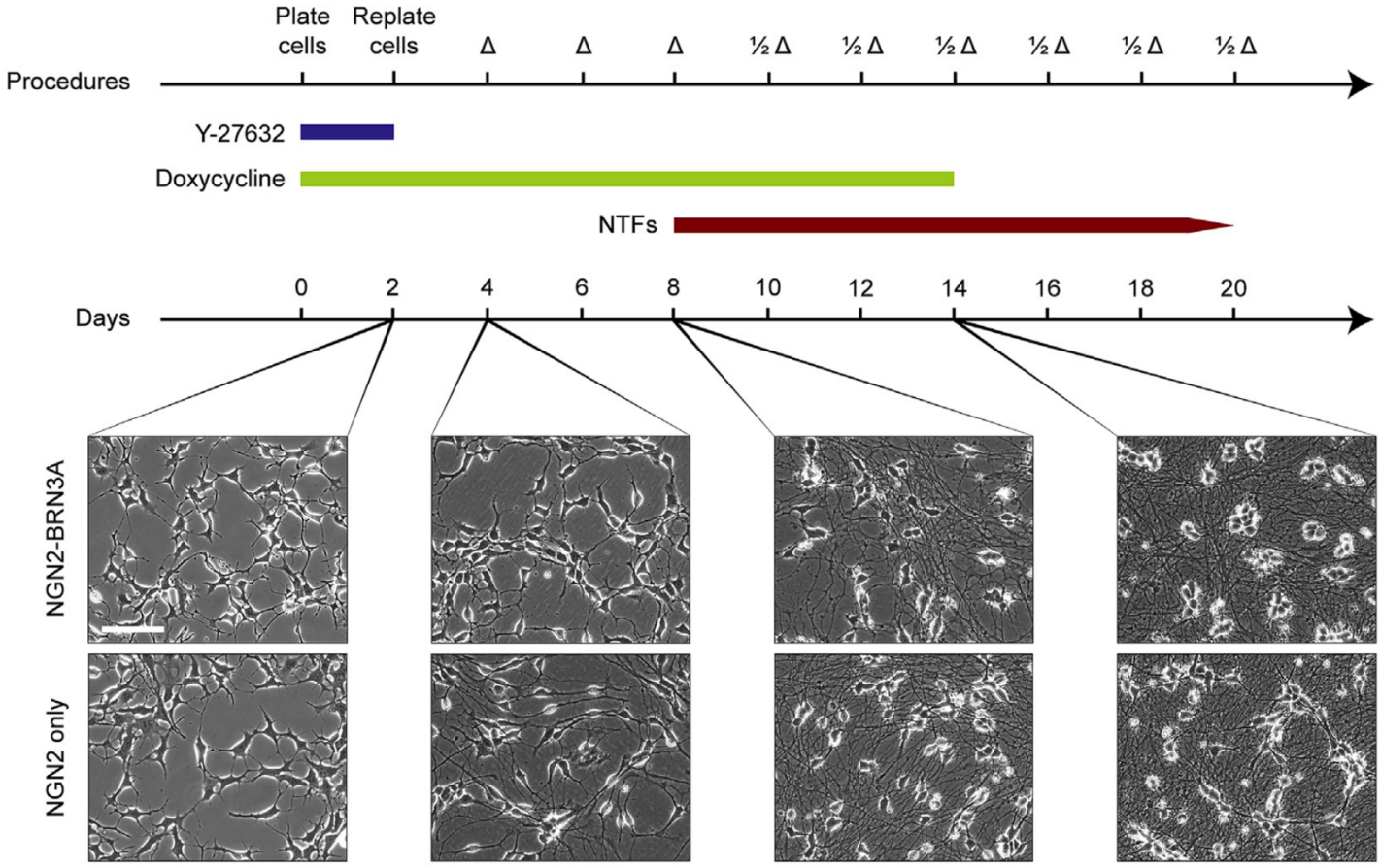
	Written by: Thu Pham, Shen Laboratory

Table 1: Media components and change schedule.

Day	Action	Media Components
D0	plate	NDM + Doxycycline (2ug/ml) + rock inhibitor (Y-27632)
D1	Δ	NDM + Doxycycline (2ug/ml)
D2	replate	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml)
D4	Δ	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml)
D6	Δ	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml)
D8	Δ	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml) + neurotrophic factors (NTFs, all 10ng/ml, including BDNF, GDNF, β-NGF and NT-3)
D10	½ Δ	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml) + NTFs
D12	½ Δ	NDM + Doxycycline (2ug/ml) + Laminin (2ug/ml) + NTFs
D14	½ Δ	NDM + Laminin (2ug/ml) + NTFs
D16	½ Δ	NDM + Laminin (2ug/ml) + NTFs
D18	½ Δ	NDM + Laminin (2ug/ml) + NTFs
D20	½ Δ	NDM + Laminin (2ug/ml) + NTFs



1. Prepare NDM media. *Table 2: NDM media preparation*

Reagent	Cat. No.	Working Conc.	500 ml	1000 ml
DMEM/F12	11330032	50%	243.75 ml	487.5 ml
Neurobasal medium	21103049	50%	243.75 ml	487.5 ml
B27 (50X)	17504044	0.5X	5 ml	10 ml
N2 (100X)	17502048	0.5X	2.5 ml	5 ml
GlutaMAX (100X)	35050061	1X	5 ml	10 ml

2. **On day 0**, coat wells with Matrigel (Corning, 354277). Wash cells once with PBS and add Accutase at 37°C and incubate for 5 mins.
3. Quench Accutase with 2x volume of DPBS. Centrifuge cells at 1000 rpm for 5 mins. Resuspend cells with 1ml NDM media + Doxycycline + rock inhibitor.
4. Count cells and plate cells in new Matrigel-coated wells at 20,000 cells/cm^2.
    - Since the cells will be replated on day 2, it does not matter which well size you use. It's best to culture cells in 6-well plates for days 0 & 1.
    - For each well in a 6-well plate, plate ~200,000 cells.
5. **On day 1**, change media completely to remove rock inhibitor. Media components: NDM + Doxy.
6. **Before day 2**, coat plates with PLO and laminin.
    - Prepare 15µg/ml PLO in PBS (+Ca +Mg) and coat the plates. Incubate overnight at 37°C.
    - The next day, aspirate PLO coating and wash once with PBS (+Ca +Mg).
    - Prepare 2µg/ml Lam in PBS (+Ca +Mg) and add to plates. Incubate overnight at 37°C. Note: In emergency cases, Lam can be incubated for 2-4hr. If PLO/Lam-coated plates are not needed right away, store in PBS at 4°C. On the day of planned use, allow the plates to equilibrate to 37 °C in the incubator before you plan to aspirate and dry them for cell seeding.

Table 3: PLO/Lam coating volume

Culture plates	Minimum coating volume
6-well	1.8 ml/well
12-well	800 µl/well
24-well	400 µl/well
48-well	200 µl/well
96-well	60 µl/well
384-well	12 µl/well
10 cm dish	10 mL/dish

7. **On day 2**, prepare NDM + Doxy + 2µg/ml Lam. Prepare PLO/Lam-coated plates by aspirating Lam coating and air dry them for 15-20 mins.
8. Wash cells with PBS and dissociate cells with Accutase (37°C, 5 mins). Quench Accutase with 2x volume of DPBS. Centrifuge cells at 1000 rpm for 5 mins. Resuspend cells with 1ml prepared media, and count cells.
9. Plate cells at 50,000 cells/cm^2. If the neurons are planned for quantified imaging experiments, plate cells at 35,000 cells/cm^2.

Table 4: Day 2 replating density

Culture plates	# cells per well for 50k/cm^2	# cells per well for 35k/cm^2
6-well	400,000-480,000	-
12-well	175,000	122,500
24-well	90,000	66,500
48-well	55,000	38,500
96-well	-	8,000
384-well	-	2,000
10 cm dish	2,800,000	-

10. **From day 3 to day 20**, change media according to Table 1.
    - Neurons can get dislodged very easily, so when changing media, pipet very slowly. If handling with 6-well and 12-well plates, use serological pipets as they are less harsh than P-1000 pipette tips.
    - After doxy treatment is terminated, media can be changed every 3 days.
11. Neurons are mature **on day 21** and can be used for experiments on this day.