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Generation of functional induced DA neurons (iDANs) from dermal fibroblasts of adult donors

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Mitochondrial antigen presentation in dendritic cells of the mouse spleen

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ASAP Collaborative Rese…

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We use this protocol and it's working

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Abstract

This describes the steps for direct DA neurons reprogramming of patient's fibroblasts.

Attachments

PDF

Direct DA reprogramm...

127KB

Guidelines

Notes

1. It is recommended to prepare the ENM and LNM fresh. Both these media can be kept up to one month at 4°C.
2. The manipulations described in this protocol must be done in a biological safety cabinet, under sterile conditions. Wearing protection including gloves and a labcoat when handling the cells is highly recommended.
3. Regular testing for mycoplasma contamination is highly recommended, as it could affect the efficiency of conversion as well as cell's growth and viability rate.
4. The choice of multi-well plate format depends on the desired neuronal yield. While the conversion efficiency varies depending on the line, a neuronal conversion of 10% of TAU+ cells and 3% of TH+ cells should be expected with this protocol.
5. Note that the neuronal markers Tuj1, MAP2, the dopaminergic marker TH as well as neuritic extensions appear early in the reprogramming process but do not necessarily mean a full neuronal conversion. Long-term cultures of the cells are needed for appearance of electrophysiologically functional neuronal properties.

Materials

Materials

1. Human dermal fibroblasts
2. Multiwell plates (Nunc)
3. T75 flasks
4. Cell counter
5. 1ml cryogenic tubes
6. Controlled-rate freezing container (ex. CoolCell LX Cell Freezing Container)
7. Disinfectant solution capable of neutralizing lentiviruses
8. Sterile 0.01% (m/V) gelatin aqueous solution
9. MEF medium (see Table 2 for composition)
10. Freezing medium (see Table 2 for composition)
11. Early neuronal medium (ENM) (see Note 1 and Table 2 for composition)
12. Late neuronal medium (LNM) (see Note 1 and Table 2 for composition)
13. lentiviral vectors containing Ascl1, Lmx1a, Lmx1b, FoxA2, Otx2 and Nurr1 and shRNA against REST (Addgene; Cat. 27150, 33013, 35001, 33014, 34997, 35000, 127573, 127574)

	A	B	C	D
	Medium name	Component	Stock concentratio	Working concentration
	MEF medium	DMEM + GlutaMAX	N/A	N/A
		Penicillin/streptomycin	10,000 U/ml	100 U/ml
		Fetal bovine serum (FBS)	N/A	10%
		Doxycycline	2 mg/ml	2 µg/ml
	Freezing medium	MEF medium	N/A	45%
		Fetal bovine serum (FBS)	N/A	45%
		DMSO	N/A	10%
	Early neuronal medium (ENM)	NDiff 227 medium	N/A	N/A
		GDNF	20 µm/ml	2 ng/ml
		NT3	10 µg/ml	10 ng/ml
		db-cAMP	50 mM	0.5 mM
		CHIR99021	10 mM	2 µM
		SB431542	20 mM	10 µM
		Noggin	100 µg/ml	50 ng/ml
		LDN-193189	10 mM	0.5 µM

	A	B	C	D
		LM-22A4	20 mM	2 µM
	Late neuronal medium (LNM)	NDiff 227 medium	N/A	N/A
		LM-22A4	20 mM	2 µM
		GDNF	20 µm/ml	2 ng/ml
		NT3	10 µg/ml	10 ng/ml
		db-cAMP	50 mM	0.5 mM

Table 2. Media composition

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

1. Seeding of fibroblasts for conversion to induced DA neurons

1 Thaw a vial containing fibroblasts in a water bath at 37 °C, until only a small piece of frozen congelation medium is remaining (See Notes 2 and 3).

2 Quickly transfer the content of the vial to a 15 ml tube containing at least 4 mL MEF medium. Centrifuge 400 x g, 00:05:00. 5m

3 Discard the supernatant and resuspend the cells in 1 mL fresh MEF medium.

4 Transfer the cell suspension to a T75 flask containing 9 mL MEF medium and put the flask in the incubator at 37 °C in [M] 5 % CO2.

5 The next day, do a full medium change with fresh MEF medium.

6 Expand the fibroblasts until they have reached about 95% confluency. Change the medium twice a week

2. Plating the cells for reprogramming

7 Coat the multi-well plate with [M] 0.1 % gelatin solution to each well. Incubate Overnight 37 °C. (See Note 4).

8 The following day, vacuum the medium of the T75 flask containing the cells. Wash once with DPBS, then dissociate the cells with 1 mL trypsin 0.05% at 37 °C for about 00:03:00, or until all the cells are detached and dissociated. 3m

9 Add 3 mL MEF medium to neutralize the trypsin, then transfer the full 4 mL to a 15 ml tube. Flush out the cells a second time with an extra 3 mL MEF medium, then transfer to the same tube.

10 Spin the cells at 400 x g, 00:05:00, then discard the supernatant. Resuspend the cells in 1 mL fresh MEF. 5m

11 Count the cells with a cell counter. To ensure a good conversion efficiency, the overall viability should not be lower than 90%.

12 Prepare a cell suspension in the appropriate volume to plate 25,000 cells/cm^2. The total volume will depend on the number of wells to be plated.

13 Vacuum the gelatin solution from the wells, then wash each well twice with DPBS.

14 Quickly, to prevent the gelatin from drying, mix the cell suspension and immediately transfer the appropriate volume to each well. Fill the remaining empty wells with sterile water to prevent evaporation during the conversion. Incubate the cells Overnight .

2d

3. Fibroblast's freezing

15 Centrifuge the remaining cell suspension (from go to step #10) at 400 x g, 00:05:00 .

5m

16 Meanwhile, prepare the required volume of freezing medium to obtain a final cell concentration of about 500,000 cells/ml.

17 Resuspend the cells in freezing medium and quickly distribute 1 mL of the suspension to each cryogenic tube.

18 Transfer the cryovials in a controlled-rate freezing container and store at -80 °C . Transfer the cryogenic tubes at -140 °C or in a liquid nitrogen tank the following day for long-term storage.

4. Viral transduction (Day 0)

19

Note

NOTE: All steps from the section 4. should be performed according to level 2 biohazard standard operating procedures.

20 Thaw at Room temperature the vials containing the lentiviral vectors containing the 6 transgenes (Ascl1, Lmx1a, Lmx1b, FoxA2, Otx2 and Nurr1) and the 2 shRNA sequences against REST that will be used for the DA neural conversion.

21 Prepare the necessary volume of MEF medium to a disposable sterile tube or bottle.

22 Calculate the volume of each virus required for a transduction at multiplicity of infection (MOI) of 5 for each vector. Use the following formula to do so:

$$volume\,of\,virus\,(\mu l) = \frac{Number\ of\ cells\ to\ infect \times MOI \times 1000\,\mu l}{Virus\,titer\,(TU/ml)}$$

23 Add the appropriate volume of each virus to the MEF medium prepared in go to step #21 . Mix by inverting the tube.

24 Vacuum the medium contained in each well. Add the appropriate volume of the virus-containing medium to each well. Incubate Overnight at 37 °C in [M] 5 % CO2 .

25 The next day (on day 1), replace the medium contained in each well with fresh, virus-free MEF medium, and incubate for 48:00:00 .

2d

Cell maintenance during reprogramming

26 On day 3, change the full MEF medium of each well for early neuronal medium (ENM).

27 Every 2nd-3rd day, change half of the early neuronal medium in each well until day 18.

28 From day 18 until the experimental endpoint is reached, change half of the medium with late neuronal medium (LNM) every 2nd-3rd days (See Note 4).