NOV 08, 2023

DOI:
dx.doi.org/10.17504/protocols.io.j8nlko6d5v5r/v1

Protocol Citation: Ching-Chieh Chou, Judith Frydman 2023. Neuronal transdifferentiation from human primary adult fibroblasts. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlko6d5v5r/v1

Protocol status: Working
We use this protocol and it's working

Created: Nov 08, 2023

Neuronal transdifferentiation from human primary adult fibroblasts

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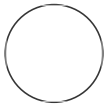

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ABSTRACT

This is a protocol for the direct conversion of human primary fibroblasts into neurons using a combination of transcription factor- and small molecule-based approach. The majority of converted neurons showing characteristics of cortical glutamatergic neurons.

Last Modified: Nov 08, 2023

PROTOCOL integer ID: 90649

Keywords: ASAPCRN, Neuron, Transdifferentiation

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-000282

Preparation of culture medium and reagents

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1. Fibroblast medium @4°C lasts for 1 month. A total of 500 mL:
   - 435 ml DMEM (High glucose, GlutaMAX) (Thermo Fisher Scientific)
   - 50 ml Fetal bovine serum (FBS) (Thermo Fisher Scientific)
   - 5 ml Penicillin-Streptomycin (Thermo Fisher Scientific)
   - 5 ml MEM NEAA (100X; Thermo Fisher Scientific)
   - 5 ml Sodium pyruvate (100 mM; Thermo Fisher Scientific)
   - 0.5 ml beta-mercaptoethanol (55mM; Thermo Fisher Scientific)
   - Sterilized by filtration through a 0.22 µm filter

2. Neuronal reprogramming medium @4°C lasts for 1 month. A total of 500 mL:
   - 240 mL DMEM/F-12 (Thermo Fisher Scientific)
   - 240 mL Neurobasal Medium (Thermo Fisher Scientific)
   - 10 mL B-27 Supplement (50X) (Thermo Fisher Scientific)
   - 5 mL N-2 Supplement (100X) (Thermo Fisher Scientific)
   - 1.25 mL GlutaMAX Supplement (Thermo Fisher Scientific)
   - 5 ml Penicillin-Streptomycin (Thermo Fisher Scientific)
   - Sterilized by filtration through a 0.22 µm filter

3. Neuronal maturation medium @4°C lasts for 1 month. A total of 500 mL:
   - 480 mL BrainPhys Neuronal Medium (STEMCELL Technologies)
   - 10 mL B-27 Supplement (50X) (Thermo Fisher Scientific)
   - 5 mL N-2 Supplement (100X) (Thermo Fisher Scientific)
   - 1.25 mL GlutaMAX Supplement (Thermo Fisher Scientific)
   - 5 ml Penicillin-Streptomycin (Thermo Fisher Scientific)
   - Sterilized by filtration through a 0.22 µm filter

4. Hexadimethrine Bromide (Polybrene) (Sigma-Aldrich)

5. Forskolin (Sigma-Aldrich)

6. Dorsomorphin (Tocris)

7. SB 431542 (Tocris)

8. XAV939 (Stemgent)

9. Doxycycline (Cayman)

10. Puromycin (Thermo Fisher Scientific)

11. BDNF Recombinant Protein (Peprotech)

12. NT-3 Recombinant Protein (Peprotech)

13. Poly-L-ornithine hydrobromide (Sigma-Aldrich)

14. Human rhLaminin-521 (Thermo Fisher Scientific)

15. Human Vitronectin (Thermo Fisher Scientific)

Transduction/induction/selection of human fibroblasts

2 **Day -1**: Plate human fibroblasts at 200K cells per well of a poly-L-ornithine coated 6-well plate.

2.1 Dilute sterile-filtered 0.01% poly-L-ornithine in PBS at a 1-to-10 ratio.

2.2 Coat the plate and incubate in a 37°C incubator for 2 hr.

2.3 Wash the plate with H2O for 5 min on a shaker and repeat for three times. Airdry the plate before plating the cells.

2.4 Rinse the cells with sterile 1x PBS to remove all complete medium.

2.5 Add 0.05% Trypsin-EDTA to cover the bottom of the dish. Incubate at 37°C with 5% CO2 for ~5 minutes. Cells should round up and become dislodged.

2.6 Neutralize trypsin-EDTA activity by adding the complete media.

2.7 Gently pipette to resuspend the cells and transfer the cell-containing medium to a 15 mL conical tube.

2.8 Centrifuge at 200xG for 5 min to pellet the cells.

2.9 Remove the supernatant. Cell numbers are determined for each sample by a hemocytometer.

2.10 Add fresh culture medium to adjust the cell concentration to 200K cells per well and each well contain 2 mL of culture medium.

3 **Day 0**: Infect cells with 2 mL of viral supernatants per well of a 6-well plate.

3.1 Make virus dilutions by adding each viral supernatant to cold Fibroblast medium. 100 to 400 µL of each viral supernatant (M2rtTA, Ngn2-puro, Ascl1, Brn2, Myt1l) depending on viral titers.

3.2 Combine these viral supernatants and add cold Fibroblast medium to reach a total of 12 mL for one 6-well plate.

3.3 Add 4 µg/mL Polybrene to the virus dilutions.

3.4 Remove old Fibroblast medium and incubate cells with virus dilutions for 24 hr.

4 **Day 1**: Remove virus-containing medium and add fresh Fibroblast medium plus 1 µg/mL doxycycline.

5 **Day 2**: Change to fresh Fibroblast medium plus doxycycline and puromycin.

6 **Day 4**: Coat glass coverslips or plates with Vitronectin/rLaminin-521 and replate the transduced cells.

> **Note**
>
> The conversion efficiency and cell survival are much better after replating as compared with no replating.

6.1 Coat with vitronectin (1:100 dilution of stock concentration 0.5 mg/mL in PBS) for 1 hr at room temperature. No need to rinse for vitronectin-coating coverslips or plates.

6.2 Coat with rhLaminin-521 (1:100 dilution of stock concentration 100 µg/mL in DPBS with Ca^{2+} and Mg^{2+}) for 2 hr at 37°C.

6.3 In parallel, trypsinize cells by 0.05% Trypsin-EDTA for 5 min at 37°C and perform PSA-NCAM selection using magnetic-based cell sorting.

6.4 Replate the PSA-NCAM+ cells in Fibroblast medium plus doxycycline. Generally ~50K cells per cm^2.

7 **Day 5**: Switch to Neuronal reprogramming medium plus small molecules.
- 5 µM Forskolin
- 2 µM Dorsomorphin
- 10 µM SB 431542
- 2 µM XAV939
- 1 µg/mL Doxycycline

8 Change half of the culture medium every 2 to 3 days.

9 **Day 12**, Switch to Neuronal reprogramming medium supplemented with small molecules and neurotrophic factors.
- 5 µM Forskolin
- 2 µM Dorsomorphin
- 10 µM SB 431542
- 2 µM XAV939
- 1 µg/mL Doxycycline
- 10 ng/mL BDNF
- 10 ng/mL NT-3

10 Change half of the culture medium every 2 to 3 days.

11 **Day 20**, switch to Neuronal maturation medium supplemented with small molecules and neurotrophic factors.
- 5 µM Forskolin
- 2 µM Dorsomorphin

- 10 µM SB 431542
- 1 µg/mL Doxycycline
- 10 ng/mL BDNF
- 10 ng/mL NT-3

> **Note**
>
> Suggest half medium change to gradually replace the neuronal reprogramming medium with maturation medium. This reduces the exposure of neurons to the air.

12 Change half of the culture medium every 3 to 4 days.

13 Neurons will mature on and after 5 weeks in culture and be ready for experiments

14 Neurons can be cultured for about 9 weeks.