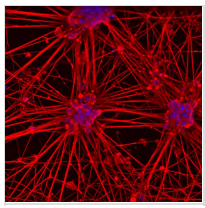

May 16, 2022

Immunocytochemistry of motor neurons derived from iPSCs with the hNIL construct protocol

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This protocol describes the immunocytochemistry for staining motor neurons derived from induced pluripotent stem cells (iPSCs) using the hNIL transgenic factors in a CLYBL safe harbor site. For the protocol on this differentiation, refer to the Clelland Lab's Differentiation of iPSCs with the hNIL construct into motor neurons protocol.

ICC of motor neurons derived from iPSCs with the hNIL construct_ClellandLab.pdf

DOI

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hNIL motor neurons, immunocytochemistry staining, pluripotent stem cells (iPSCs), ICC, immunofluorescence staining, motor neuron staining, immunofluorescence

protocol ,

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Mar 30, 2022 Maria Sckaff University of California, San Francisco

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Equipment used in the immunocytochemistry staining of motor neurons derived from hNIL iPSCs.

A	B	C
Equipment	**Manufacturer**	**Catalog Number**
Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid	Corning	353219
Stovall Life Science, The Belly Dancer	Fisher Scientific	15-453-211
VWR Mini Shaker	Avantor/VWR	12620-938

Reagents used in the immunocytochemistry staining of motor neurons derived from hNIL iPSCs.

A	B	C
Equipment	**Manufacturer**	**Catalog Number**
4% PFA	Alfa Aesar	J61899
1X DPBS, no Ca, no Mg	ThermoFisher	14-190-235
Bovine Serum Albumin (BSA)	Sigma Aldrich	A4503-50G
Triton X-100	any	any
DAPI (4'-6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher	D1306

6-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid
Microplate
Falcon® **353219**

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The Belly Dancer™ Orbital Platform Shaker
Shaker

IBI Scientific™ **15-453-211**

Mini Shakers
Shakers

VWR® **12620-938**

Other suggested materials:

- Parafilm
- Sodium Azide

beta Tubulin Monoclonal Antibody (AA10) **Thermo Fisher Scientific Catalog #480011**

Immunocytochemistry of the hNIL motor neurons: Day 1: Fixing, Permeabilizing, Blocking and Coating Cells with Primary Antibody

2h 50m

30m

1

This protocol can be followed at any time after Day 7 in the hNIL differentiation of iPSCs into motor neurons protocol. This protocol has been optimized to neurons plated on 96-well plates.

Without removing media from the wells, add 4% PFA on all target wells and let it sit for **00:30:00** at **Room temperature** (the volume of 4% PFA should equal the volume of media already in the well, resulting in a final well concentration of 2% PFA).

2 Discard the media and PFA by carefully and gently tapping the plate upside down onto absorbent wipes (KIMTECH Kimwipes are appropriate). Discard the Kimwipes in designated PFA waste.

If fixing and staining need to be performed on separate days, after step 2, wash the plate

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thrice with 1X DPBS, **00:10:00** each time at slow agitation on the Belly Dancer. Then store the plate at **4 °C** , with the sides covered by parafilm to avoid evaporation. Only permeabilize the cells (step 3) on the day that the cells will be incubated with primary antibody.

3

Wash cells with 1X DPBS + 0.1% Triton-X ("DPBS-T" at **100 µL** per well) to permeabilize the cells.

3.1 Briefly wash cells with 1X DPBS + 0.1% Triton-X ("DPBS-T" at **100 µL** per well). Discard the spent wash onto absorbent wipes. (1/3)

3.2 Wash cells with 1X DPBS + 0.1% Triton-X ("DPBS-T" at **100 µL** per well) for 10m **00:10:00** at slow agitation on the Belly Dancer. Discard the spent wash onto absorbent wipes. (2/3)

3.3 Wash cells with 1X DPBS + 0.1% Triton-X ("DPBS-T" at **100 µL** per well) for 10m **00:10:00** at slow agitation on the Belly Dancer. Discard the spent wash onto absorbent wipes. (3/3)

4

Block the target wells with 1X DPBS-T + 5% BSA at **Room temperature** for **01:00:00** (**50 µL** /well). 1h

Do not exceed this time to avoid over blocking your neurons.

5 Discard blocking agent onto an absorbent wipe, then add primary antibodies diluted in 1X DPBS-T + 5% BSA (**50 µL** /well) at the appropriate concentrations.

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It is advisable to include the beta tubulin monoclonal primary antibody (1:250 from ThermoFisher Scientific Catalog # 480011) to show neuronal morphology.

6  1h

Incubate at **4 °C** **Overnight** on a plate shaker, with the sides covered by parafilm to avoid evaporation.

Immunocytochemistry of the hNIL motor neurons: Day 2: Coating Cells with Secondary Antibody

1h 50m

7 

Discard the primary antibody solution from each well using a multichannel pipette.

Ensure to never touch the bottom of the well and always pipette slowly to not lift neurons from the well bottom.

8 

Wash with 1X DPBS-T to remove any unbound primary antibody (**100 µL** /well). First briefly, then twice for 10 minutes each time at slow agitation on the Belly Dancer.

8.1 Wash with 1X DPBS-T briefly. (1/3)

8.2 Wash with 1X DPBS-T for **00:10:00** at slow agitation on the Belly Dancer. 10m (2/3)

8.3 Wash with 1X DPBS-T for **00:10:00** at slow agitation on the Belly Dancer. 10m (3/3)

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9 Add desired secondary antibodies diluted in 1X DPBS-T + 5% BSA. Incubate at **Room temperature** for **01:00:00** in the belly dancer. 1h

10 

Wash once briefly with 1X DPBS-T (**100 µL** /well).

11  10m

Wash with 1X DPBS-T with DAPI (1:1000-1:10,000) for **00:10:00** (100 µL/well).

12 

Wash with 1X DPBS (100 µL/well).

12.1 Wash with 1X DPBS for **00:10:00** . (1/2) 10m

12.2 Wash with 1X DPBS for **00:10:00** . (2/2) 10m

13 Change the media to 1X DPBS or 1X DPBS + Sodium Azide 0.02%, if storing long term, (**100 µL** /well) and store at **4 °C** .

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