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*.

Immunocytochemistry of the hNIL motor neurons

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.

Day 1: Fixing, Permeabilizing, Blocking and Coating Cells with Primary Antibody.

1. Without removing media from the wells, add 4% PFA on all target wells and let it sit for 30 minutes at RT (the volume of 4% PFA should equal the volume of media already in the well and will be a final concentration of 2% PFA)
2. Discard the media and PFA by carefully tapping the plate upside down onto absorbent wipes (KIMTECH Kimwipes are appropriate). Discard the Kimwipes in designated PFA waste.
    a. If fixing and staining need to be performed on separate days, after step 2, wash the plate thrice with 1X DPBS, 10 minutes each time at slow agitation on the Belly Dance. 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 stained
3. Wash cells with 1X DPBS + 0.1% Triton-X ("DPBS-T" at 100 µL/well) thrice to permeabilize the cells. First briefly, then twice for 10 minutes each time at slow agitation on the Belly Dancer. Discard the spent washes each time onto absorbent wipes
4. Block the target wells with 1X DPBS-T + 5% BSA at RT for 1 hour (50 µL/well). Do not exceed this time to avoid overblocking your neurons
5. Discard blocking agent onto an absorbent wipe, then add primary antibodies in 1X DPBS-T + 5% BSA (50 µL/well) at the appropriate concentrations. It is advisable to include the beta tubulin monoclonal primary antibody (1:250 from *ThermoFisher Scientific* Catalog # 480011) to show neuronal morphology
6. Incubate at 4°C overnight on a plate shaker, with the sides covered by parafilm to avoid evaporation

Day 2: Coating Cells with Secondary Antibody.

1. Discard the primary antibodies onto absorbent wipes such that the solution from each well falls as an individual stream from the plate to avoid cross contamination
    a. If there is a concern of antibody cross contamination, the solution from each well can be removed using a multichannel pipette as appropriate, ensuring to never touch the bottom of the well and always pipette slowly to not lift neurons from the well bottom
2. Wash with 1X DPBS-T thrice to remove the unbound primary antibody (100 µL/well). First briefly, then twice for 10 minutes each time at slow agitation on the Belly Dancer
3. Add secondary antibodies in 1X DPBS-T + 5% BSA. Incubate at RT for 1 hour in the belly dancer.
4. Wash once briefly with 1X DPBS-T (100 µL/well)
5. Wash with 1X DPBS-T with DAPI (1:1000-1:10,000) for 10 minutes (100 µL/well)
6. Wash with 1X DPBS twice for 10 minutes each time (100 µL/well)
7. 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

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

Table 1: Equipment used in the immunocytochemistry staining of motor neurons derived from hNIL iPSCs.