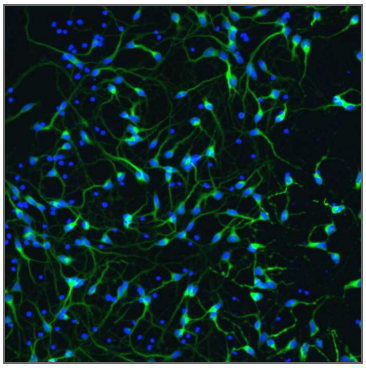

VERSION 2

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Immunocytochemistry for the characterization of hiPSC to Motor Neuron differentiation V.2

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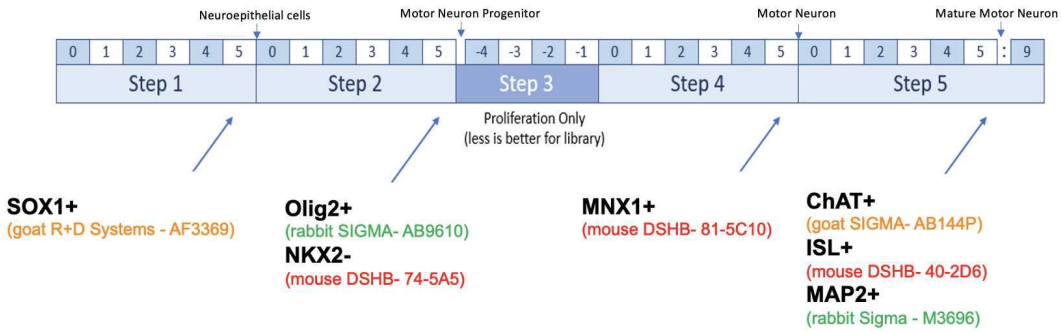
ABSTRACT

This immunocytochemistry protocol is used for the characterization of IPSC differentiation into motor neurons using several biomarkers: neuroepithelial cells (SOX1), motor neuron progenitors (OLIG2 and NKX2.2), motor neurons (MNX1), and the mature motor neurons (ISL, ChAT, MAP2).

*Primary and secondary antibody information located in materials section



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

Created: Jan 23, 2024

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PROTOCOL integer ID: 94029

MATERIALS

- BlockAid™ Blocking Solution **Thermo Fisher Catalog #B10710**
- Phosphate-buffered saline (PBS, 1X), sterile-filtered **Thermo Scientific Catalog #J61196.AP**
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML**
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4612**
- 32% Paraformaldehyde **Electron Microscopy Sciences Catalog #50-980-495**

Primary Antibody Stains and Concentrations

Step 1:

- **SOX1** (R+D Systems - AF3369 - 100 ug) - **Reconstitute** in 500uL of sterile 1xPBS makes 200 ug/ul concentration - Use
  15 ug/ml ****Add 75ul to 1ml BSA**** - Needs Anti-Goat Secondary

Step 2:

- **OLIG2** (Sigma - AB9610-100ul volume in tube-0.5 mg/ml,) - Use 1.2 ug/mL (1:400 dilution)
  ****Add 2.5ul to 1ml BSA**** - Needs Anti-Rabbit Secondary

- **NKX2** (DSHB - 74.5A5 - 1ml total volume in tube - 23 ng/ul) - Use 2ug/ml total
  ****Add 86ul to 1ml BSA**** - Needs Anti-Mouse Secondary

Step 4:

- **MNX1** (DSHB - 81.5C10 - 1ml total volume in tube - 36 ng/ul) - Use 2ug/ml total
  ****Add 55ul to 1ml BSA**** - Needs Anti-Mouse Secondary

- **MNX1 (2nd option) (**Novus Biological- NBP224691- 0.1 mg/ml) - Use 2ug/ml total
  Add 4ul to 1mL BSA- Needs Anti-Rabbit Secondary

Step 5:

- **MAP2** (Sigma - M3696-100ug in tube - 1.0 mg/mL) - Use 2.5 ug/mL (1:400 dilution)
  ****Add 2.5ul to 1ml BSA**** - Needs Anti-Rabbit Secondary

- **MAP2 (2nd option)** (Thermo Scientific- PA5-17646 - 100 uL in tube - 73.6 µg/mL) - Use 0.74 ug/mL (1:100 dilution) ****Add 10ul to 1ml BSA**** - Needs Anti-Rabbit Secondary

- **CHAT** (Sigma - AB144P - 500ul - Concentration: > = 0.1 - < 1%) - Use 1 ug/mL (1:100 dilution)
  ****Add 10ul to 1ml BSA**** - Needs Anti-Goat Secondary

- **ISL1** (DSHB - 40.2D6 - 1ml total volume in tube - 28 ng/ul) - Use 2 ug/ml total
  ****Add 70ul to 930ul BSA**** - Needs Anti-Mouse Secondary

Secondary Antibody Stains and Concentrations

(Use volumes are based on a total volume of 1ml 3% BSA staining solution. Volumes of Primary or Secondary antibodies should be subtracted from 1ml volume, ie. 150ul SOX1 added to 850ul BSA = 1ml BSA total. Adjust volumes as needed for staining solutions)

- Alexa Fluor Plus 488 Donkey Anti-Rabbit IgG (H+L) (ThermoFisher A32790- 1 mg in tube - 2 mg/mL stock) -
  - Use 2 ug/ml total ****Add 1ul to 1ml BSA****

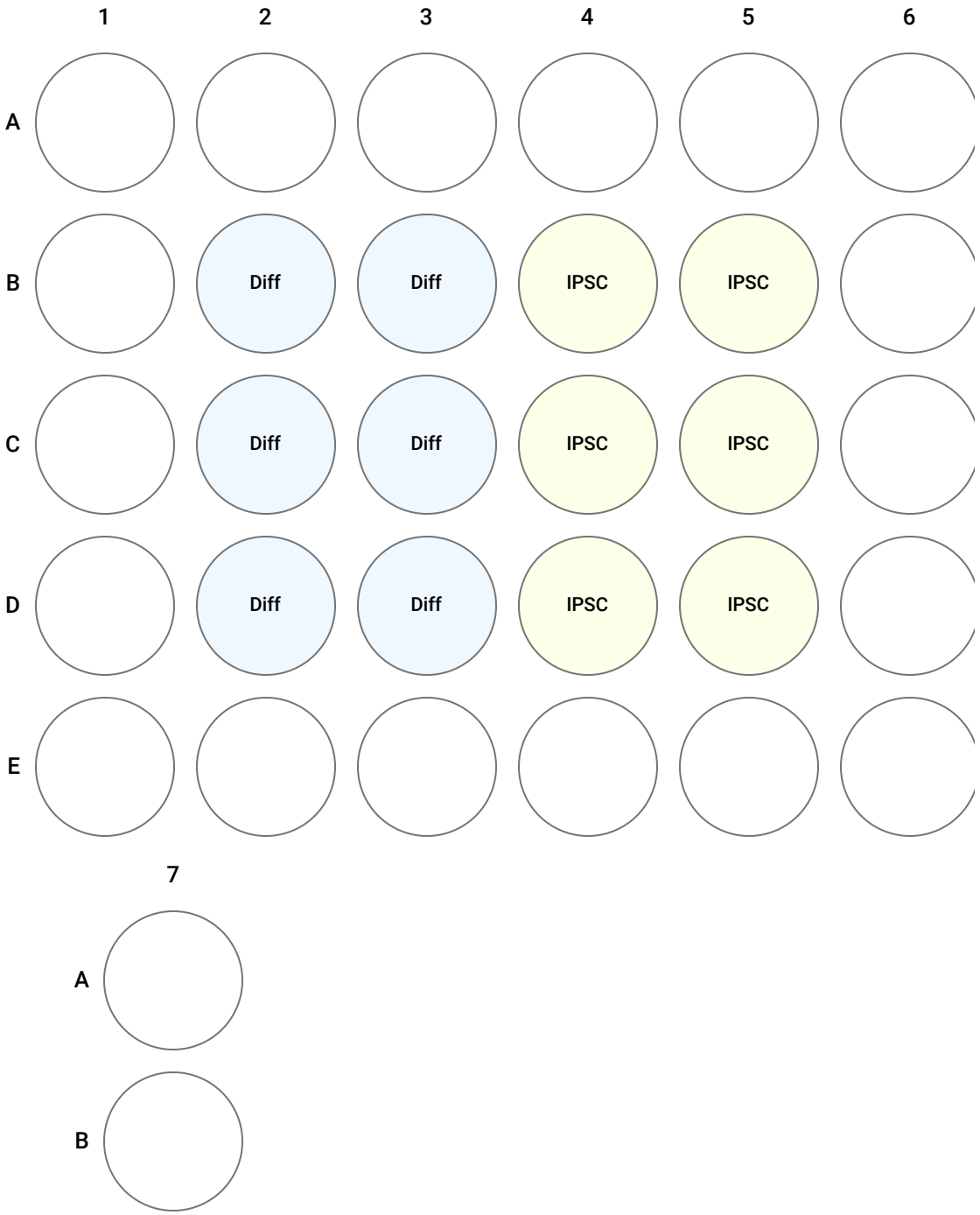
- Alexa Fluor 555 Donkey Anti-Goat IgG (H+L) (ThermoFisher A21432 - 1 mg in tube - 2 mg/ml stock)
  - Use 10 ug/ml total ****Add 5ul to 1ml BSA****

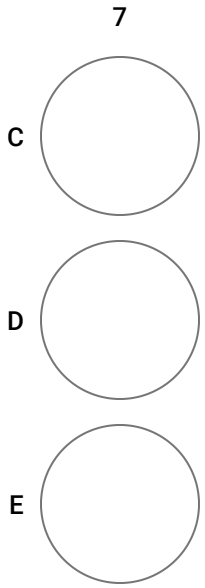
- AlexaFluor 647 Donkey Anti-Mouse IgG (H+L) (ThermoFisher A31571 - 1mg in tube - 2 mg/ml stock)
  - Use 2 ug/ml total ****Add 1ul to 1ml BSA****

BEFORE START INSTRUCTIONS

To ensure that the observed results are not just random events, use controls, such as undifferentiated IPSCs, to accurately analyze that the antibodies are working as expected.

Here is an example of how we seed cells onto a 96-well plate. The 96-well has an equal amount of wells of differentiated (blue) and undifferentiated cells (yellow) for analysis. Cells are also counted and seeded at equal densities.





1 Remove the medium from your cells

Note

Tip the vessel towards you and pipette from the bottom corner of the well

2 FIX - Dilute 32% Paraformaldehyde solution to 4% PFA in 1X phosphate-buffered saline (PBS)

3 Add 100uL of 4% PFA to each well in the 96-well plate. (1ml if using a 6-well plate). Incubate for 00:15:00 at room temperature. 15m

4 Remove the fixative solution and wash with 1XPBS at 100ul per well using a multichannel pipette. Repeat 3 times.

Note

- Aspirate and dispense fixative and wash very slowly, so as to not dislodge your cells.
- Move the plate from side to side between each wash
- The fixed sample can be stored, covered in foil, for several days at 4°C if needed.

5 PERMEABILIZE - Add 100uL of 0.5% Triton X-100 to each well of a 96-well (1ml if using 6-well plate)

6 Incubate for 00:15:00 at room temperature. 15m

7 Remove the permeabilization solution and wash 3 times with 1XPBS

8 BLOCK - Add 100ul of 3% BSA (bovine serum albumin) or blockAid-blocking solution to each well of a 96-well plate slowly to Block. (1ml if using 6-well plate)

9 Incubate for at least 01:00:00 (up to overnight) at room temperature. 1h

10 Calculate the amount of primary antibody needed (located in materials section) and dilute in 3% BSA + 0.3% Triton X-100 solution

11 PRIMARY ANTIBODIES - Remove 3% BSA from wells and add 100uL of primary antibody per well

Note

Make sure primary and secondary antibodies are stored properly. Aliquots should be put in the -20 and thawed once, with any remainder kept at 4°C.

12 Incubate for 01:00:00 at room temperature in a dark place or overnight at 4°C. 1h
(Different primary antibody stains may take longer to stain and could require optimization).

13 Remove primary antibody and wash three times slowly with 1xPBS

Note

- Remember to aspirate and dispense fixative and wash very slowly, so as to not dislodge your cells and continue moving plate side to side between washes.

14 SECONDARY ANTIBODIES - Calculate amount of secondary antibody needed (located in materials section) and dilute in 3% BSA+ .3% TritonX-100 solution and 1:4000 Hoechst.

15 Add 100uL per well in 96-well and incubate for at least 01:00:00 at room temperature in a dark place.

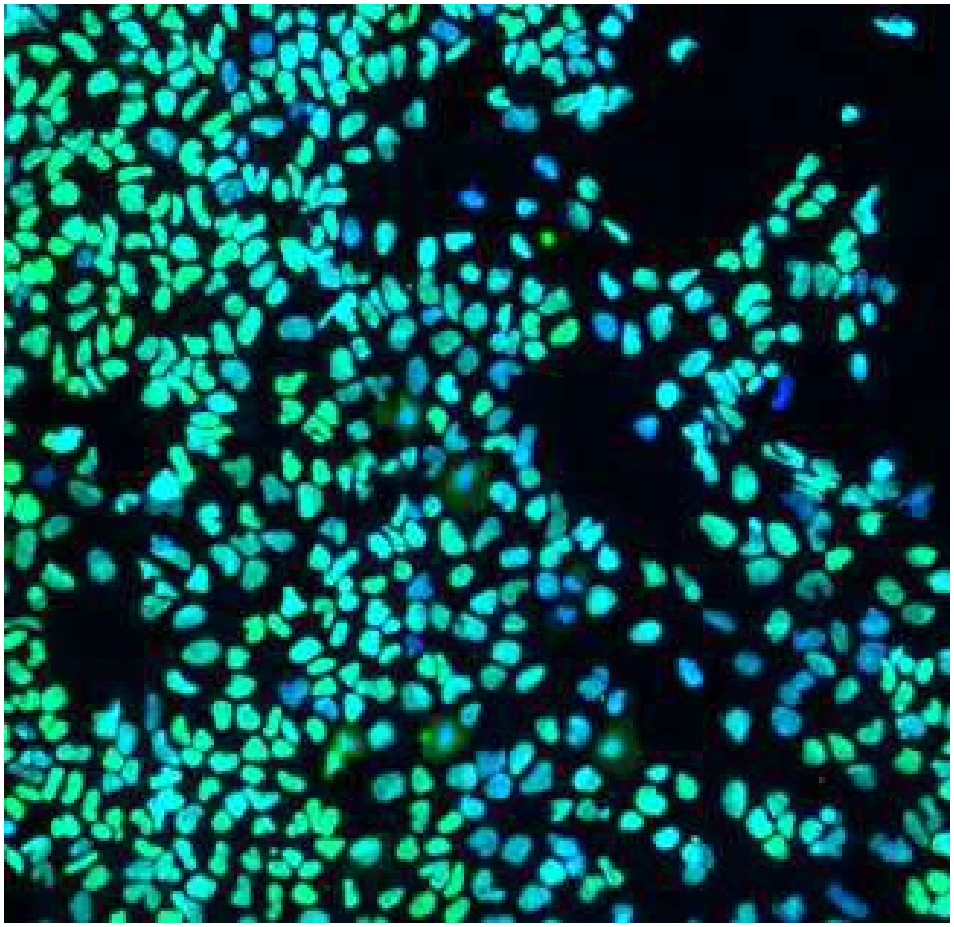
16 Remove secondary antibody and wash three times with 1xPBS

17 Scan on the confocal microscope

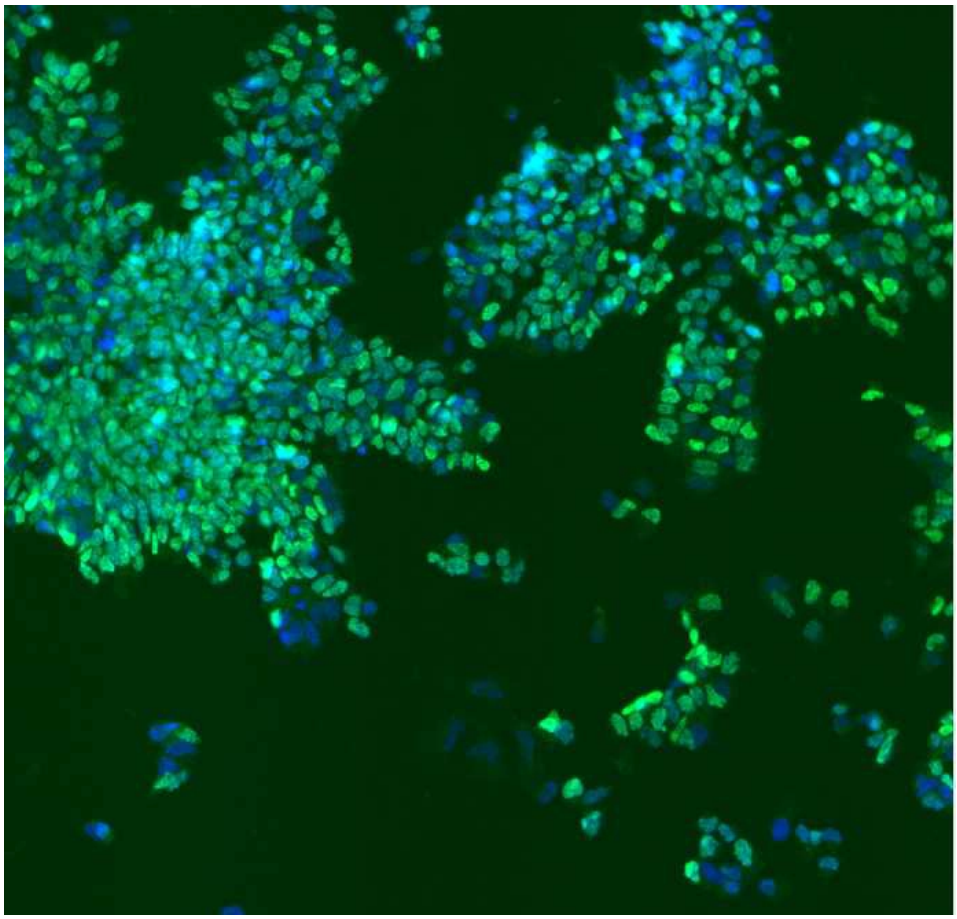
Note

We currently use the ImageXpress Confocal HT.ai High-Content imaging system and the InCarta image analysis software to quantify the percent of live nuclei that are positive for each stain and measure intensity levels of each antibody.

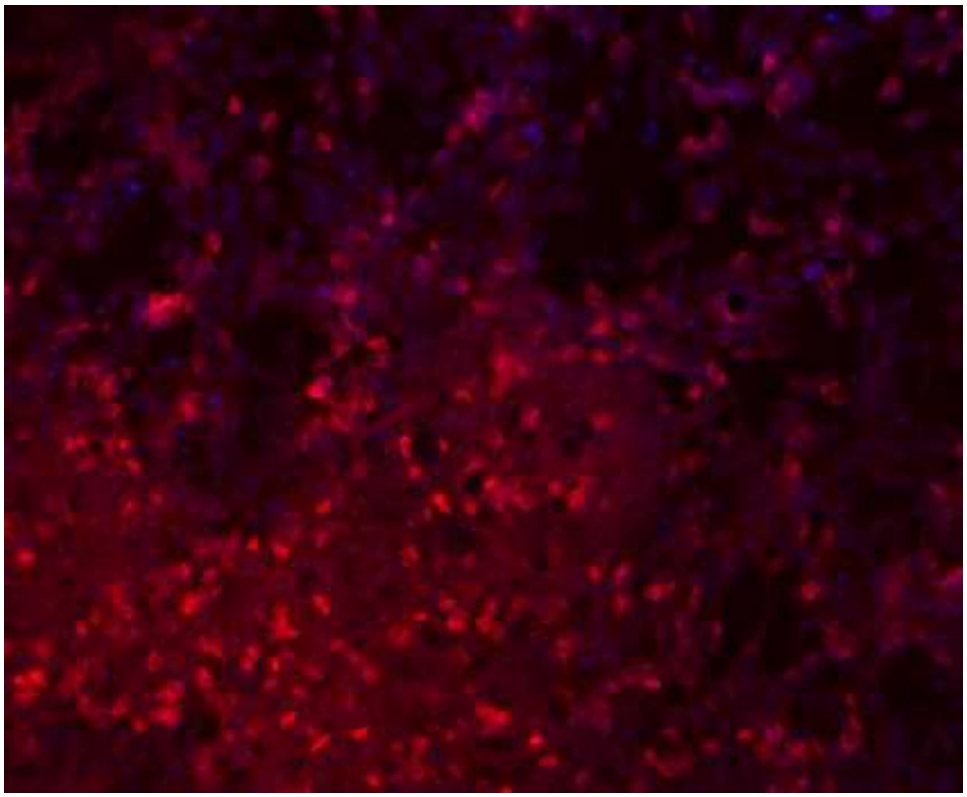
Expected result



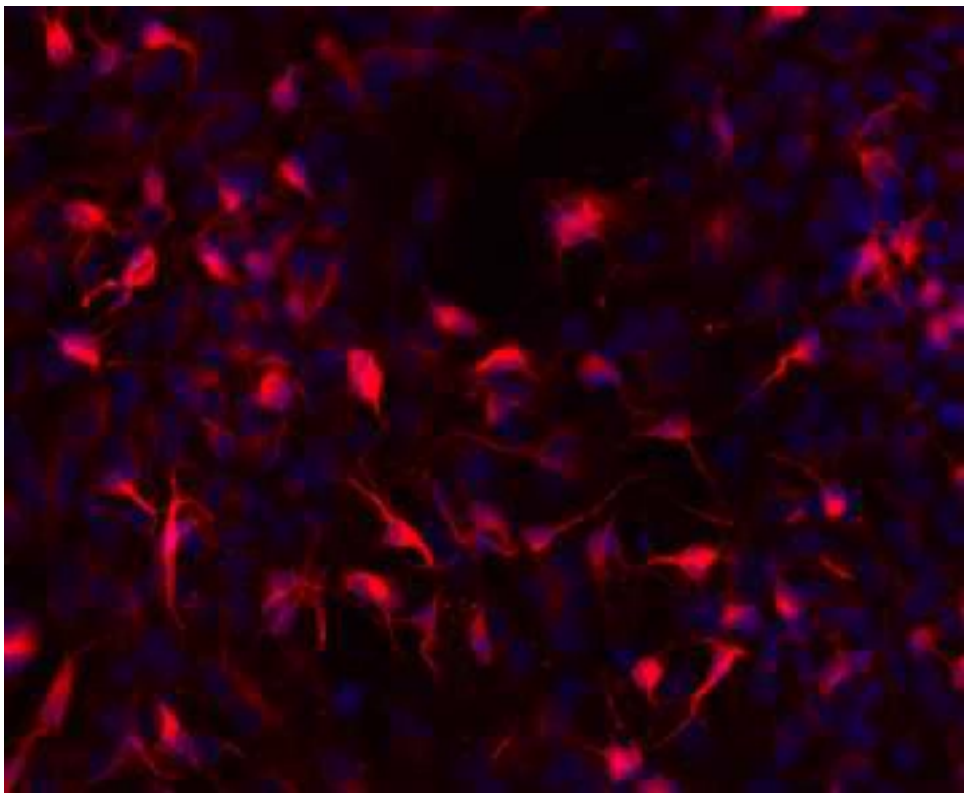
Neuroepithelial cells stained with SOX1 (green) and Hoechst (blue)



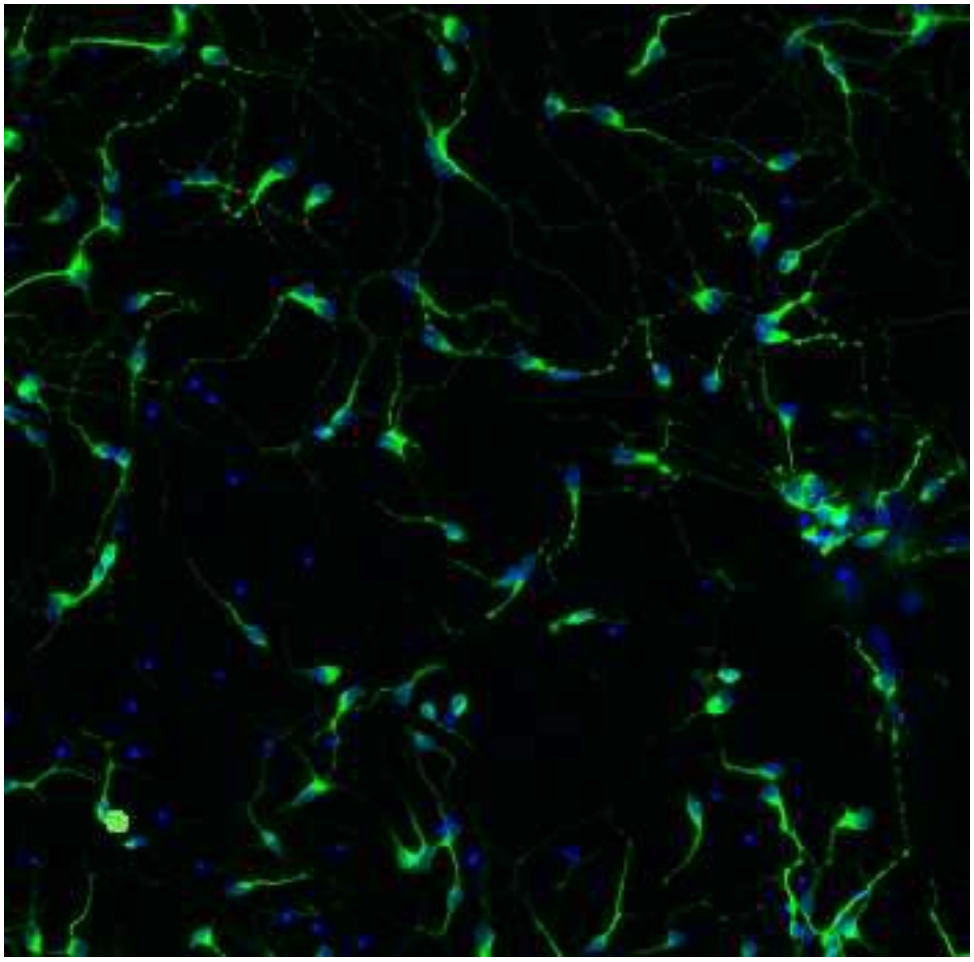
Neural progenitors Stained with Olig2 (green) and Hoechst (blue)



Motor neuron stained with MNX1 (red) and Hoechst (blue)



Mature motor neurons stained with ISL(red) and Hoechst (blue)



Mature motor neurons stained with Map2 (green) and Hoechst (blue)