# Differentiation of iPSCs with the hNIL construct into motor neurons protocol

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This protocol describes the differentiation of induced pluripotent stem cells (iPSCs) into motor neurons using the hNIL transgenic factors in a CLYBL safe harbor site.

This protocol is based on hNIL differentiation protocol by Fernandopulle *et al.* from Michael Ward's lab[1].

## Maintenance and Preparation of the iPSCs for the hNIL differentiation into motor neurons

Maintaining iPSCs

iPSCs should be cultured for at least 2-3 passages after initial hNIL transfection or from frozen stocks before starting a motor neuron differentiation. iPSCs that have been recently thawed or are otherwise stressed (e.g., recently nucleofected) can result in phenotypically abnormal motor neurons and poor differentiation. See Flores *et al.* for complete iPSC culture methods[2]. Briefly, we maintain iPSCs on Matrigel-coated plastic culture dishes (Growth Factor Reduced Matrigel diluted in 50 mL of KnockOut DMEM to a concentration of 80 µg/mL) with mTeSR™ Plus medium. Media should be changed every other day, and can be passaged every 4-5 days with ReLeSR™ (see below). ROCK1 inhibitor can be added at 10 µM in media to freshly passaged iPSCs for 1 day to limit spontaneous differentiation.

Passaging iPSCs[2]

iPSCs can be passaged as clumps (for routine expansion of iPSC cultures) or single cells (for differentiation). For routine expansion, wash the well with DPBS -Ca/-Mg (1-2mL/well*) then add 1mL/well* of ReLeSR™ for 60 seconds. Aspirate most of the ReLeSR™, but do not overdry, and incubate the wells at room temperature for 3-4 minutes. Add fresh mTeSR™ Plus media to the well (1mL/well*) and pipette gently to detach the iPSCs and break colonies into small clumps. Clumps should be visible by eye. A confluent well can be split 1:6 to 1:20 depending on the desired confluency and rate of growth of the iPSC line. If desired, before replating, cells can be centrifuged at 800rpm for 15 seconds to pellet clumps and remove any single cells (which remain in suspension). ROCK1 inhibitor is not necessary when clump passaging, but can be added at 10 µM (final concentration in well solution) for 1 day to limit spontaneous differentiation.

For differentiation (or anytime cell counting is needed), wash the well with DPBS -Ca/-Mg (1-2mL/well*) then add 0.5 mL* of Accutase®. Incubate the plate for 5 minutes at 37°C. After the incubation, tap the plate to fully detach the iPSCs, then add 0.5 mL* of KnockOut DMEM with 20% FBS

(alternately, 2.5 mL/well* of DPBS +Ca/+Mg can be used in place of DMEM/FBS). Transfer the cells and solution to a conical vial and centrifuge for 3 minutes at 800 rpm to pellet the cells. Aspirate the supernatant carefully as to not disturb the cell pellet, and resuspend the cells in mTeSR™ Plus with ROCK1 inhibitor at 10 µM. ROCK1 inhibitor is highly recommended when passaging iPSCs as single cells to prevent spontaneous differentiation.

* These volumes are based on experiments run on a 6-well plate and are scalable to other cell culture vessels.

**hNIL differentiation into motor neurons**

Day 0:

1. Coat the receiving vessel with Matrigel at least 30 minutes before starting Day 0 (but no more than 24-36 hours prior to starting) and incubate at 37°C
2. Bring the mTeSR™ Plus with ROCK1 inhibitor (final concentration of 10uM in well, 1000X dilution from 10mM stock) and the Accutase® to room temperature
3. Aspirate the spent media and wash the wells with cells once with 1X DPBS -Ca/-Mg
4. Add appropriate volume of Accutase® per well
5. Incubate the plate for 5 minutes at 37°C
6. Remove plate from the incubator and tap the plate to release the cells
7. Quench the Accutase® using five times the volume DPBS +Ca/+Mg (if you used 0.5 mL of Accutase®, quench with 2.5 mL of DPBS)
8. Transfer the cells to a conical vial
9. Centrifuge the vial at 800 rpm for 3 minutes
10. Aspirate the Accutase® with DPBS, being careful not to disturb the cell pellet at the bottom of the vial
11. Resuspend the cells with an appropriate volume of mTeSR™ Plus with ROCK1 inhibitor (final concentration of 10uM in well, 1000X dilution from 10mM stock). Aim for roughly 1 mL of mTeSR™ Plus with ROCK1 inhibitor per 1 million cells
12. Pipette up and down to mix well and produce an evenly distributed solution
13. Count the number of cells using a Countess II (any preferred equivalent way of counting cells is also appropriate)
    a. Countess II count parameters:
        i. Size 0 to 30
        ii. Brightness 0 to 255
        iii. Circularity 80
        iv. Auto Lighting selected
14. Aspirate the Matrigel from the receiving vessel
15. Add appropriate volume of media (the mTeSR™ Plus with ROCK1 inhibitor at 10uM) to the receiving vessel
16. Add volume of cell mixture appropriate for the number of cells desired in the well. For a 10cm dish, we have found 1-2 million iPSCs at day 0 produces 3-5 million cells at day 3.
17. Place the receiving vessels (which now has your cells) back in the incubator at 37°C
18. Shake the plate in all four directions to ensure the cells are evenly distributed in the well

| Cell culture vessel | Matrigel, PDL, and laminin in KO DMEM volume per well | DPBS volume for washes per well | Accutase® volume per well |
|---|---|---|---|
| 96-well plate | 120 µL | 150 µL | 50 µL |
| 24-well plate | 250 µL | 400 µL | 125 µL |
| 12-well plate | 500 µL | 1 mL | 250 µL |
| 6-well plate | 1 mL | 2 mL | 500 µL |
| 10-cm dish | 4 mL | 6 mL | 3 mL |
| 15-cm dish | 12 mL | 18 mL | 9 mL |

*Table 1*: Volumes of coating solutions (Matrigel, PDL, and laminin in KO DMEM), DPBS, and Accutase® for each cell culture vessel.

Day 1:

1. Prepare the neural induction medium (***NIM***) as follows:
    a. DMEM/F12 97 mL
    b. N-2 1 mL
    c. NEAA 1 mL
    d. GlutaMAX 1 mL
2. Calculate the volume of freshly prepared NIM needed to perform a full media change (see Table 2) and aliquot in a vial of appropriate size
3. Add the appropriate factors for day 1 to the NIM aliquot you aliquoted in step 2:

| | Factor | Final Concentration | Stock | Dilution |
|---|---|---|---|---|
| a. | ROCK1 inhibitor | 10 µM | 10 mM | 1:1,000 |
| b. | Doxycycline | 2 µg/mL | 2 mg/mL | 1:1,000 |
| c. | Compound E | 0.2 µM | 2 mM | 1:10,000 |

4. Aspirate the spent media from the wells with cells
5. Add the appropriate volume of media to each well
6. Place the plate with cells in fresh media back in the incubator at 37°C

Day 2:

1. Select the receiving cell-culture vessel. This is the plate on which your neurons will grow permanently (see recommendations below)
2. Coat the wells which will be receiving cells on day 3 with the appropriate volume of 1X PDL at 0.1 mg/mL (see Table 1)
3. Place the coated plate back in the incubator at 37°C

*Recommendations for protein or RNA extraction*: A minimum surface area equivalent to three 6-wells is recommended per RNA or protein sample to be extracted. Regular cell-culture plates (plastic, flat bottom) are well-suited for this application.

*Recommendations for imaging purposes*: We recommend high quality plastic plates, such as the Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate (see Table 4) due to improved

long-term neuron attachment. Glass-bottom plates or coverslips may also be used, but in our hands, about 20-50% of the wells experience lifting as the neurons age past day 14 in culture. Glass-bottom plates are recommended when the neurons will be fixed for imaging at an earlier stage, such as between day 7 and day 14 in culture. Premature detachment can be minimized by very gentle handling, slow pipetting, and never completely removing the media from the wells (including when PFA fixing, see below).

*Note on PDL pre-coated plates*: If using a PDL pre-coated plate, you will only need to rehydrate it on day 3 in DPBS once prior to coating receiving wells with laminin and the seeding of the cells.

Day 3:

*Part 1 (if using PDL pre-coated plates, rinse once with DPBS and skip to step 4)*

1. Aspirate out the 1X PDL from the receiving cell-culture vessel
2. Wash the receiving wells with 1X DPBS twice
3. Let receiving wells dry in the laminar flow hood for 20-30 minutes
4. Coat receiving cell-culture vessel with the appropriate volume of NIM with laminin mouse protein (at a concentration of 15 µg/mL) for 2-5 hours at 37°C (see Table 1 for volume)

*Part 2*

1. Prepare quenching media DMEM/F12 with 20% FBS at an appropriate volume. Per well in a 6-well plate, 0.5 mL of quenching media will be needed
2. Calculating the volume of day 3 NIM media needed
    a. When calculating the volume of day 3 NIM media needed, consider that you will need the volumes in Table 2, as well as 1 mL per 1 million cells resuspended before seeding.
    b. That is, if you have about 3 million cells that you will seed on 2 wells of a 6-well plate and 72 wells of a 96-well plate, you will need roughly 3 mL + 4 mL + 10.8 mL = 17.8 mL of day 3 media. However, you should always prepare about 10% more to account for loss and round up, which in this case would be 20 mL of day 3 NIM media.
3. Preparing day 3 ***NIM*** media:

| | Factor | Final Concentration | Stock | Dilution |
|---|---|---|---|---|
| a. | ROCK1 inhibitor, | 10 µM | 10 mM | 1:1,000 |
| b. | Doxycycline, | 2 µg/mL | 2 mg/mL | 1:1,000 |
| c. | Compound E, | 0.2 µM | 2 mM | 1:10,000 |
| d. | Laminin, | 1 µg/mL | 1 mg/mL | 1:1,000 |
| e. | BrdU, | 40 µM | 40 mM | 1:1,000 |

*Recommendation to minimize the chances of mitotically-active cells overwhelming the culture*: Include Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well at every media change starting at day 3. This is particularly useful if maintaining the motor neurons for more than four weeks and for imaging purposes.

4. Aspirate the spent media and wash the wells with cells once with 1X DPBS -Ca/-Mg
5. Add appropriate volume of Accutase® per well (0.5 mL/well in a 6-well plate)

6. Incubate the plate for 5 minutes at 37°C
7. Remove plate from the incubator and tap the plate to release the cells
8. Quench the Accutase® with DMEM/F12 + 20% FBS, at a volume equal to the volume of Accutase® per well (if you used 0.5 mL of Accutase®, quench Accutase® with 0.5 mL of DMEM/F12 + 20% FBS). DPBS +Ca/+Mg is also suitable.
9. Pipette the solution gently around the well thoroughly to ensure cells have dissociated from the well bottom
10. Move the cells from their wells to a 15 mL vial (or vial of appropriate volume depending on how much volume of cells you have)
11. Centrifuge the vial at 800 rpm for 3 minutes
12. Aspirate the Accutase® with DMEM/F12 + 20% FBS, being careful not to disturb the cell pellet at the bottom of the vial
13. Resuspend the cells with an appropriate volume of day 3 NIM media (about 1 mL per 1 million cells in the vial)
14. Count the cells using a Countess™ (or preferred counting method) using the same parameters from Day 0
15. Aspirate the NIM with 15 µg/mL laminin from each receiving well
16. Plate appropriate number of cells per well using the appropriate media volume per well depending on your receiving cell culture vessel

- Sample calculation and coating advice for uniform seeding of cells onto receiving vessel:
    - Calculate the volume of day 3 NIM media needed per well
    - Multiply that volume by the desired concentration of cells per well (e.g., 20k cells/well or 1 x $10^6$ cells/well)
    - Divide that number by the concentration found by counting using the Countess™ (e.g. 2 x $10^6$ cells/well)
    - The resulting number is the volume you need from the cell solution you resuspended in day 3 NIM media in step 13
    - Add that volume to your final desired volume of day 3 NIM media (e.g., 4 mL if seeding onto 2 wells of a 6-well plate)
    - Mix well to ensure a uniform distribution of cells
    - Seed appropriate media volume (which now has cells as well) onto the receiving cell culture vessel
    - This is particularly important when seeding cells onto small wells (e.g., 96-well plates), where a uniform distribution of cells can improve survival rates.

17. Carefully place the plate with cells back in the incubator at 37°C, rocking the plate briefly after placing the plate back, such that the cells are uniformly distributed onto the well

<u>Day 4</u>:

1. Prepare the day 4 ***NIM*** media for performing **a full-media change**

| | Factor | Final Concentration | Stock | Dilution |
|---|---|---|---|---|
| a. | B-27 Supplement, | | 50x | 1:50 |

| | | | | |
|---|---|---|---|---|
| b. | Culture One Supplement, | | 100x | 1:100 |
| c. | Laminin, | 1 µg/mL | 1 mg/mL | 1:1,000 |
| d. | BDNF, | 20 ng/mL | 20 µg/mL | 1:1,000 |
| e. | GDNF, | 20 ng/mL | 20 µg/mL | 1:1,000 |
| f. | NT3, | 20 ng/mL | 20 µg/mL | 1:1,000 |

*In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.*

2. Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir
3. Add the fresh media slowly at a **direction normal to the well side wall**. Never add the fresh media directly onto the well bottom, which will produce a shear force capable of lifting (and consequently killing) the neurons
4. Place the plate back in the incubator at 37°C

Day 7:

1. The risk of lifting only increases as the motor neurons age and mature on the well bottom.
    a. Proper care is needed to ensure the neurons neither dry out nor lift; consider increasing the volume of media per well, starting on day 7 by up to 50%.
    b. That is, if you had 150 µL/well in a 96-well plate, then consider maintaining 200 µL/well from now on, by aspirating 50 µL/well on day 7, and adding back 100 µL/well of fresh day 7 media.
2. Prepare the day 7 ***NIM*** media for performing **a *half*-media change**

    *Note on growth factors BDNF, GDNF, and NT3 concentrations*: although this is a half-media feed, we provide the full amount of growth factors, so **their dilution doubled**. **This doubling of the growth factor dilution is maintained during all half-media changes from here.**

| | **Factor** | **Final Concentration** | **Stock** | **Dilution** |
|---|---|---|---|---|
| a. | B-27 Supplement, | | 50x | 1:50 |
| b. | Culture One Supplement, | | 100x | 1:100 |
| c. | Laminin, | 500 ng/mL | 1 mg/mL | 1:1,000 |
| d. | BDNF, | 20 ng/mL | 20 µg/mL | **1:500** |
| e. | GDNF, | 20 ng/mL | 20 µg/mL | **1:500** |
| f. | NT3, | 20 ng/mL | 20 µg/mL | **1:500** |

*In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.*

3. Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir
4. Add the fresh media slowly in at a direction normal to the well side wall (which is easier by tilting the plate about 60-degrees towards you). Never add the fresh media directly onto the well bottom, which will produce a shear force capable of lifting (and consequently killing) the motor neurons
5. Place the plate of motor neurons back in the incubator at 37°C

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Day 10 on:

1. Prepare Neurobasal Medium (***NMM***) as follows:
    a. Neurobasal 97 mL
    b. N-2 1 mL
    c. NEAA 1 mL
    d. GlutaMAX 1 mL
2. Prepare the day 10 ***NMM*** media for performing **a *half*-media change**

| | Factor | Final Concentration | Stock | Dilution |
|---|---|---|---|---|
| a. | B-27 Supplement, | | 50x | 1:50 |
| b. | Culture One Supplement, | | 100x | 1:100 |
| c. | Laminin, | 500 ng/mL | 1 mg/mL | 1:1,000 |
| d. | BDNF, | 20 ng/mL | 20 µg/mL | **1:500** |
| e. | GDNF, | 20 ng/mL | 20 µg/mL | **1:500** |
| f. | NT3, | 20 ng/mL | 20 µg/mL | **1:500** |

*In addition, consider including Aphidicolin (a reversible polB inhibitor) at a final concentration of 5 µM in the well, if appropriate for your application.*

3. Aspirate the media slowly from the wells, using a pipette, discarding into a reservoir
4. Add the fresh media slowly in at a direction normal to the well side wall (which is easier by tilting the plate about 60-degrees towards you)
5. Place the plate of motor neurons back in the incubator at 37°C
6. Perform a ***half*-media change** with NMM and factors every 7 days for up to 9 weeks or more (if using the plate for imaging) or every 4 days (if using the plate for protein, RNA, or DNA extraction) *

*The frequency of half-media changes was optimized for our genes of interest and applications and is recommended to be optimized for different genes and applications.

| Cell culture vessel | Surface area ($cm^2$) | Media volume per well | Day 3 Seeding density for ICC applications | Day 3 Seeding density for protein, RNA, DNA extraction |
|---|---|---|---|---|
| 96-well plate | 0.32 | 150 µL | 20-25k cells | - |
| 24-well plate | 1.9 | 500 µL | 80-100k cells | - |
| 12-well plate | 3.5 | 1 mL | $2 \times 10^5$ cells | - |
| 6-well plate | 9.6 | 2 mL | $0.5 \times 10^6$ cells | $1 \times 10^6$ cells |
| 10-cm dish | 56.7 | 10 mL | - | $5 \times 10^6$ cells |
| 15-cm dish | 145 | 30 mL | - | $15 \times 10^6$ cells |

*Table 2*: Number of cells and volume of media matrix needed on the *day-3 replating* in the differentiation of hNIL iPSCs into motor neurons based on the size and format of the cell culture vessel used, as well as the application. Modification from the Kampmann's Lab protocol[3].

| Item | Manufacturer | Catalog Number |
|---|---|---|
| KnockOut DMEM | ThermoFisher Scientific | 10829018 |
| Growth Factor Reduced Matrigel | Corning | 356231 |
| mTeSR™ Plus and supplement | Stemcell Technologies | 1000276 |
| ReLeSR™ | Stemcell Technologies | 05872 |
| Gibco™ DPBS, no Ca, no Mg | ThermoFisher Scientific | 14-190-235 |
| Gibco™ DPBS, Ca, Mg | ThermoFisher Scientific | 14-040-117 |
| Accutase | Stemcell Technologies | 07920 |
| DMEM/F-12, HEPES | ThermoFisher Scientific | 11330032 |
| N-2 Supplement | ThermoFisher Scientific | 17502048 |
| MEM Non-Essential Amino Acids Solution (NEAA) | ThermoFisher Scientific | 11140050 |
| GlutaMAX Supplement | ThermoFisher Scientific | 35050061 |
| Culture One Supplement | ThermoFisher Scientific | A3320201 |
| B-27 Supplement, serum free | ThermoFisher Scientific | 17504044 |
| y-Secretase Inhibitor XXI, Compound E | Millipore Sigma | 565790 |
| Poly-D-Lysine (PDL) | ThermoFisher Scientific | A3890401 |
| Laminin Mouse Protein, Natural | ThermoFisher Scientific | 23017015 |
| Doxycycline Hyclate (reconstituted in water) | Millipore Sigma | D3447 |
| 5-Bromo-2'-deoxyuridine (BrdU) | Millipore Sigma | B9285 |
| ROCK1 Inhibitor (Y-27632 2HCl) | Selleckchem | S1049 |
| Neurobasal | Life Technologies | 21103-049 |
| HyClone Characterized Fetal Bovine Serum (FBS) | Cytiva | SH30071.03HI |
| Recombinant Human BDNF | Peprotech | 450-10 |
| Recombinant Human GDNF | Peprotech | 450-02 |
| Recombinant Human NT3 | Peprotech | 450-03 |
| Aphidicolin | Millipore Sigma | 89458 |

*Table 3:* Items needed for the creation of the media and reagents used in the differentiation of hNIL iPSCs into motor neurons.

**Notes on some items that need to be reconstituted**

*Compound E*: 1 mg is reconstituted in 255 µL of ethanol and 255 µL of DMSO to make a 10,000 stock, then aliquoted and stored at -20°C for up to 6 months, minimizing exposure to light.

*Doxycycline*: diluted in cell culture grade water to 2 mg/mL and stored at -20°C (long-term storage) or 4°C (short-term storage), minimizing exposure to light.

*BrdU*: reconstituted in water to a stock of 40 mM (12.284 mg/mL).

*BDNF, GDNF, and NT3*: 50 µg reconstituted in filtered 1X DPBS with 0.1% BSA, then aliquoted and stored at -20°C for up to 3 months.

| Equipment | Manufacturer | Catalog Number |
|---|---|---|
| Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid | Corning | 353219 |
| Eppendorf® Centrifuge 5810/5810R | Millipore Sigma | EP022628168 |
| Invitrogen Countess™ II automated cell counter | ThermoFisher Scientific | AMQAX1000 |

*Table 4:* Equipment used in the differentiation of hNIL iPSCs into motor neurons.

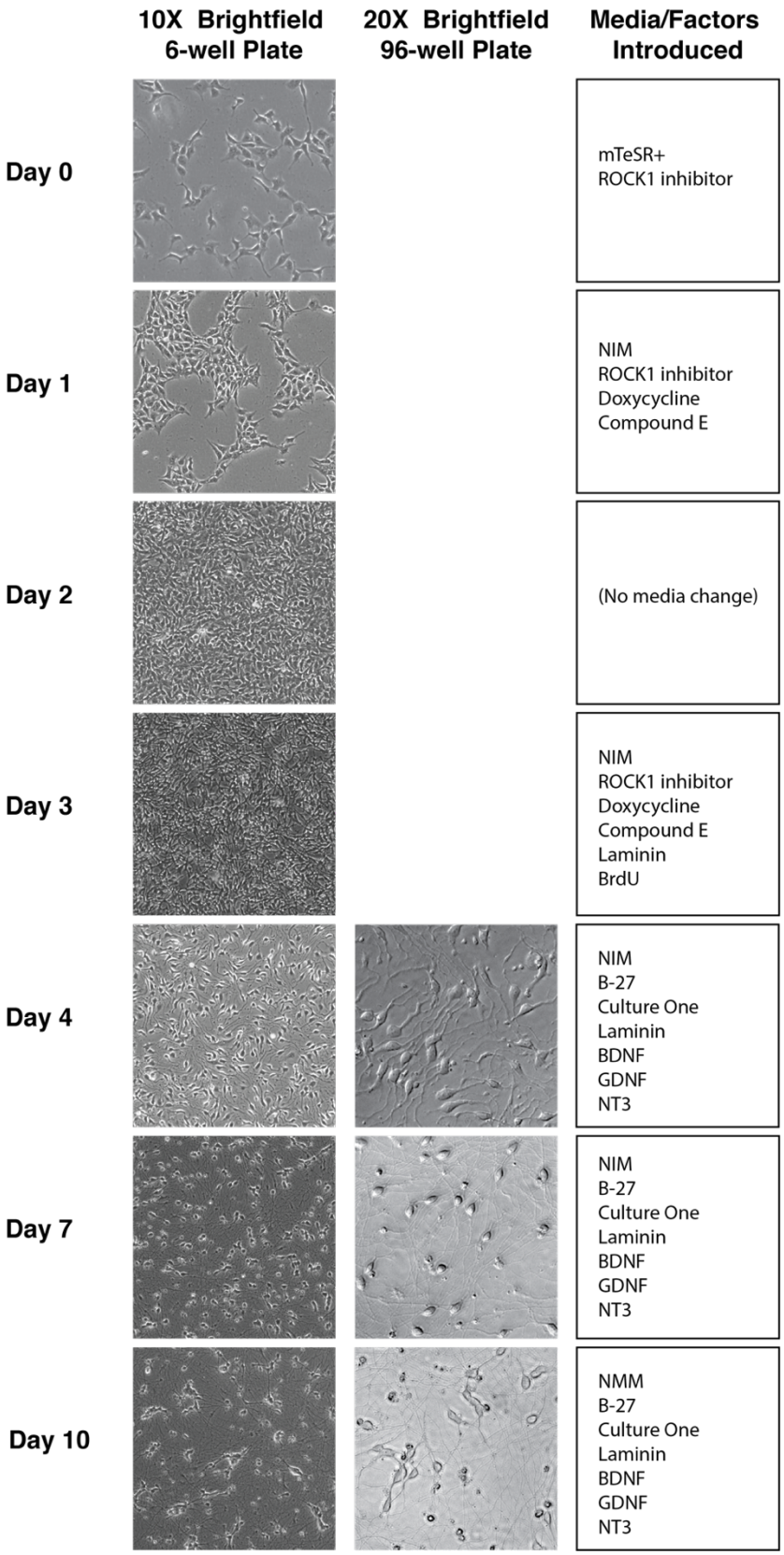


*Figure*: hNIL differentiation of iPSCs into motor neurons of WTC line initially on a 6-well plate (10X), then transferred to a 6-well plate and a 96-well plate (20X) after day-3 differentiation

**References**

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