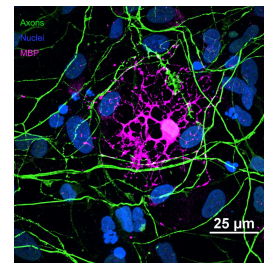


Jul 22, 2024

🌐 Generation of Myelinating Oligodendrocytes from Pluripotent Stem Cells

DOI

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Protocol status: In development

We are still developing and optimizing this protocol

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Keywords: Oligodendrocyte lineage induction, Progenitor cells, hESC cell, Magnetic labelling, hiPCS cells, generation of myelinating oligodendrocyte, myelinating oligodendrocyte, oligodendrocyte progenitor cell, motor neuron differentiation protocol at day, motor neuron differentiation protocol, astrocyte, pluripotent stem cells this document, pluripotent stem cell, motor neuron, cells ready at the same time, day 27 of the motor differentiation, motor differentiation, cell, opcs in pdgf media, separate differentiation protocol

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Abstract

This document contains two separate differentiation protocols, one to generate oligodendrocyte progenitor cells (OPCs) and the other to generate motor neurons. For co-culture, both protocols must be done simultaneously to have cells ready at the same time. Starting the motor neuron differentiation protocol at day 38 of the OPC protocol is recommended. Alternatively, motor neurons can be frozen at day 14 and thawed to prepare for the co-culture, which occurs on day 27 of the motor differentiation. Do not maintain OPCs in PDGF media for more than two weeks after sorting as they have the propensity to differentiate into astrocytes.

Note

This is a step-by-step protocol that was adapted from Douvaras and Fossati, 2015 (PMID: 26134954), with modifications.

Guidelines

Differentiation to OLIG2+ progenitors (Timing 12 d)

This is a step-by-step protocol that was adapted from Douvaras and Fossati, 2015(PMID: 26134954), with a few modifications.

Generating motor neurons for co-culture (Timing 27 d)

Adapted from Ziller and Ortega, 2018 (PMID: 29551301) with a few modifications from Gabriella Robertson (Gama lab).

Setting up and maintaining co-culture (Timing 20 d)

Co-culture protocol was adapted from García-León et al., 2018 (PMID: 29337119).

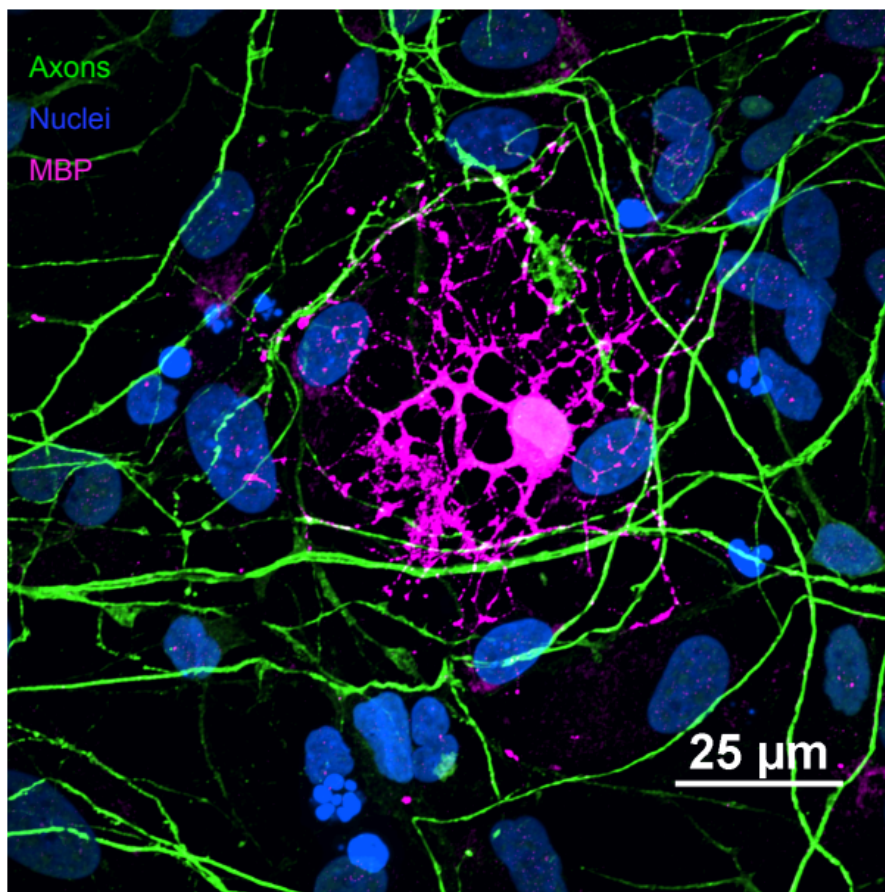


Figure 1. Representative image of a myelinating oligodendrocyte co-cultured with motor neurons. Image was taken with a Nikon spinning disc confocal microscope.



Materials

MACS[®] Separators and MicroBeads to isolate OPCs (Timing 1-4hrs)

Reagents:

- 0.5% BSA made in 1X PBS
 1. Make fresh each time and keep at  4 °C
 2.  20 mL are more than enough
 3. Make sure to vacuum filter
- DNase ( 5 µL)
- MicroBeads
 1. This protocol uses anti-A2B5 MicroBeads. Each MicroBead has varying incubation times and volumes needed for separation. Miltenyi Biotec data sheet provides a source of conditions for specific MicroBeads.
- [M] 10 millimolar (mM) Y27632 Stock

Materials:

- MiniMACS[™] separator
 1. The magnet and the metal stand are kept on the shelf in TC above the microscope. Place it in the hood and turn on the UV light for  00:30:00 to an  01:00:00 to sterilize the equipment.
- Columns (MS) for MiniMACS[™] separator (Miltenyibiotec Product Information)
- Cell Strainer (40µm)

Recipes:

Basal media:

Note

Sterile filter and store at  4 °C .



Reagent	Manufacturer	Catalog number	Final concentration
DMEM/F12	Thermo Fisher Scientific	11320033	N/A
MEM Non-Essential Amino Acids Solution	Thermo Fisher Scientific	11140050	1X
GlutaMAX Supplement	Thermo Fisher Scientific	35050061	1X
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122	1X
2-Mercaptoethanol	Bio-Rad	1610710	55 μ M
Total	N/A	N/A	N/A

NIM (Neural Induction Media):

Note

Store at 4 °C.

Reagent	Manufacturer	Catalog number	Final concentration
Basal Medium	N/A	N/A	N/A
Insulin	Millipore Sigma	I9278	25 μ g/mL
SB431542*	Reprocell	04-0010-10	10 μ M
LDN193189*	Reprocell	04-0074	250 nM
RA*	Millipore Sigma	1610710	100 nM
Total	N/A	N/A	N/A

Add small molecules fresh daily*N2 medium:**

Note

Store at 4 °C.

Reagent	Manufacturer	Catalog number	Final concentration
Basal Medium	N/A	N/A	N/A
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
SAG*	STEMCELL Technologies	73412	1 µM
RA*	Millipore Sigma	1610710	100 nM
Total	N/A	N/A	N/A

**Add small molecules fresh daily*

N2B27 medium:

Note

Store at 4 °C.

Reagent	Manufacturer	Catalog number	Final concentration
Basal Medium	N/A	N/A	N/A
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
B27 minus Vitamin A supplement	Thermo Fisher Scientific	12587010	1X
SAG*	STEMCELL Technologies	73412	250 nM
RA*	Millipore Sigma	1610710	100 nM
Total	N/A	N/A	N/A

**Add small molecules fresh daily*

PDGF medium:

Note

Store at 4 °C.

Reagent	Manufacturer	Catalog number	Final concentration
Basal Medium	N/A	N/A	N/A
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
B27 minus Vitamin A supplement	Thermo Fisher Scientific	12587010	1X
PDGFaa	R&D Systems	221-AA-025	10 ng/ml
IGF-1	Peprtech	100-11	10 ng/ml
HGF	Peprtech	315-23	5 ng/ml
NT-3	Peprtech	450-03	10 ng/ml
T3	Sigma	T2877	60 ng/ml
Biotin	Sigma	B4639	100 ng/ml
cAMP	Sigma	D0627	1 µM
Insulin	Millipore Sigma	I9278	25 µg/ml

MNIM (Motor Neuron Induction Media):

Note

Sterile filter. Store at 4 °C . *Add small molecules fresh daily



Reagent	Manufacturer	Catalog number	Final concentration
DMEM/F12 [#]	Thermo Fisher Scientific	11320032	N/A
Neurobasal media [#]	Thermo Fisher Scientific	21103049	1X
MEM Non-Essential Amino Acids Solution [#]	Thermo Fisher Scientific	11140050	1X
GlutaMAX Supplement [#]	Thermo Fisher Scientific	35050061	1X
Penicillin-Streptomycin [#]	Thermo Fisher Scientific	15140122	1X
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
B27 minus Vitamin A supplement	Thermo Fisher Scientific	12587010	1X

SAG [*]	STEMCELL Technologies	73412	1 uM
RA [*]	Millipore Sigma	1610710	1 uM
SB431542 [*]	Reprocell	04-0010-10	10 μ M
LDN193189 [*]	Reprocell	04-0074	100 nM

MNMM (Motor Neuron Maturation Media):

Note

#Sterile filter. Store at  4 °C .



Reagent	Manufacturer	Catalog number	Final concentration
Neurobasal media [#]	Thermo Fisher Scientific	21103049	1X
MEM Non-Essential Amino Acids Solution [#]	Thermo Fisher Scientific	11140050	1X
GlutaMAX Supplement [#]	Thermo Fisher Scientific	35050061	1X
Penicillin-Streptomycin [#]	Thermo Fisher Scientific	15140122	1X
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
B27 minus Vitamin A supplement	Thermo Fisher Scientific	12587010	1X
Ascorbic Acid	Sigma	A4403	0.2 ug/mL
CNTF	R&D Systems	257-NT-010	10 ng/mL
BDNF	Peprotech	450-02	10 ng/mL
GDNF	R&D Systems	212-GD-010	10 ng/mL

Co-culture Media:

Note

#Sterile filter. Store at  4 °C .



Reagent	Manufacturer	Catalog number	Final concentration
Neurobasal media [#]	Thermo Fisher Scientific	21103049	1X
MEM Non-Essential Amino Acids Solution [#]	Thermo Fisher Scientific	11140050	1X
GlutaMAX Supplement [#]	Thermo Fisher Scientific	35050061	1X
Penicillin-Streptomycin [#]	Thermo Fisher Scientific	15140122	1X
N-2 supplement 100X	Thermo Fisher Scientific	17502048	1X
B27 minus Vitamin A supplement	Thermo Fisher Scientific	12587010	1X
Ascorbic Acid	Sigma	A4403	20 ug/mL
CNTF	R&D Systems	257-NT-010	10 ng/mL
BDNF	Peprtech	450-02	10 ng/mL
GDNF	R&D Systems	212-GD-010	10 ng/mL
T3	Sigma	T2877	60 ng/ml
Biotin	Sigma	B4639	100 ng/ml
cAMP	Sigma	D0627	1 μ M
Insulin	Millipore Sigma	I9278	25 μ g/ml

☒ DMEM/F12 Thermo Fisher Scientific Catalog #11320033

☒ 2-Mercaptoethanol Bio-Rad Laboratories Catalog #1610710

☒ Anti-Adherence Rinsing Solution STEMCELL Technologies Inc. Catalog #07010

☒ Insulin solution human Merck MilliporeSigma (Sigma-Aldrich) Catalog #I9278

☒ Stemolecule™ SB431542 Reprocell Catalog #04-0010-10

☒ Stemolecule LDN-193189 Reprocell Catalog #04-0074

☒ N-2 Supplement (100X) Thermo Fisher Catalog #17502048

☒ SAG STEMCELL Technologies Inc. Catalog #73412

☒ B27 supplement minus vitamin A Gibco - Thermo Fisher Scientific Catalog #12587010

☒ Recombinant Human PDGF-AA Protein R&D Systems Catalog #221-AA-025

☒ Recombinant Human IGF-I peprtech Catalog #100-11

☒ Recombinant Murine HGF peprtech Catalog # 315-23

☒ Recombinant Human NT-3 peprtech Catalog #450-03

☒ 3,3',5-Triiodo-L-thyronine Merck MilliporeSigma (Sigma-Aldrich) Catalog #T2877



- ⊗ Biotin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B4639-1G**
- ⊗ Dibutyl cAMP **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0627**
- ⊗ Neurobasal Medium (1x) **Gibco - Thermo Fisher Scientific Catalog #21103049**
- ⊗ MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Scientific Catalog #11140050**
- ⊗ GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #35050061**
- ⊗ Penicillin-Streptomycin **Gibco - Thermo Fisher Scientific Catalog #15140122**
- ⊗ L-Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4403**
- ⊗ Recombinant Human CNTF **R&D Systems Catalog #257-NT-010**
- ⊗ Recombinant human BDNF **peprotech Catalog #450-02**
- ⊗ Recombinant Human GDNF **R&D Systems Catalog #212-GD-010**

Before start

This document contains two separate differentiation protocols, one to generate oligodendrocyte progenitor cells (OPCs) and the other to generate motor neurons. For co-culture, both protocols must be done simultaneously to have cells ready at the same time. Starting the motor neuron differentiation protocol at day 38 of the OPC protocol is recommended. Alternatively, motor neurons can be frozen at day 14 and thawed to prepare for the co-culture, which occurs on day 27 of the motor differentiation. Do not maintain OPCs in PDGF media for more than two weeks after sorting as they have the propensity to differentiate into astrocytes.



Day -3: Replating hESCs

9m

- 1 When the cells reach 70–90% confluency, remove the medium and add  1 mL of accutase.
- 2 Incubate the plate in a  37 °C incubator for  00:05:00 .
- 3 Dilute the accutase solution by adding  2 mL of DMEM/F12 medium.
- 4 Use a cell lifter to remove cells from well and gently pipette the mixture 2–5 times with the p1000 pipette to fully dissociate the hESC cell colonies to single cells.
- 5 Transfer the cells in a conical tube.
- 6 Centrifuge the cells at  200 x g, Room temperature, 00:04:00 .
- 7 Resuspend the cell pellet in  1 mL of mTeSR1 containing  10 micromolar (μM) Y27632 and count the cells with a hemocytometer.
- 8 Plate 8×10^4 cells per well on a Matrigel-coated six-well plate with mTeSR1 supplemented with  10 micromolar (μM) Y27632.

5m



4m



Day -2: Maintaining hESCs

- 9 Aspirate and add fresh mTeSR1 media to remove Y27632.

Day -1: Maintaining hESCs

- 10 Add fresh mTeSR1 media.



Day 0: Neural induction

- 11 By now colonies should have grown evenly and reached 80% confluency. When cells have reached this point, proceed with differentiation.
- 12 Aspirate medium and add NIM to induce differentiation.
- 13 Change NIM daily until cells have reached day 8.

Day 8: Oligodendrocyte lineage induction

- 14 Aspirate medium and add N2 medium to start directing cells to oligodendrocyte lineage commitment. Change media daily until cells reach day 12.

Day 12: Cell detachment and formation of OLIG2-enriched aggregates (Timing 18 d)

11m

- 15 Prepare low-attachment plate:
 - 15.1 Rinse wells of a 6-well plate with anti-adherence solution (StemCell Tech Cat: 07010).
 - 15.2 Add N2B27 medium to a total of  3 mL in each well.
 - 15.3 Put plate aside until aggregates are ready.
- 16 Remove the old medium from differentiation plate and add  1 mL of fresh N2B27 medium per well of a six-well plate.
- 17 Use a cell lifter and place it perpendicular to the bottom of the well.





- 18 Press the cell lifter against the bottom of the plate and create a cut to the cell layer. Create at least 20 such lines parallel to each other, to cover the whole well.
 - 19 Turn the well 90° and repeat Step 17.
 - 20 Turn the well 45° and repeat Step 17.
 - 21 Use the same cell lifter to detach the remaining adherent cells by scraping the whole well.
 - 22 With a p1000 pipette, gently pipette the clumps of cells 3–5 times, and transfer the contents of one well into two wells of an ultra-low-attachment six-well plate. Feed cells every 2 days.
 - 23 Feeding:
 - 23.1 Transfer the medium containing the cell aggregates to a 15-ml conical tube and wait for 00:03:00 – 00:05:00 for the aggregates to sink to the bottom of the tube. 5m
 - 23.2 Aspirate two-thirds of the medium and replenish it with fresh N2B27 medium.
 - 23.3 Gently pipette five times up and down with a p1000 pipette.
- Note**

(!) It is important to break apart the aggregates that stick to each other through gentle pipetting.
- 23.4 Return the aggregates to the same ultra-low-attachment plate and redistribute an approximately equal number of aggregates in each well with a p1000 pipette.
 - 24 On day 20, transfer the aggregates to a 15-ml conical tube and wait for 00:03:00 for the aggregates to sink to the bottom of the tube. 3m



25 Remove two-thirds of the medium and replenish it with PDGF medium.

26 Gently pipette five times up and down with a p1000 pipette.

Note

(!) It is important to break apart the aggregates that stick to each other through gentle pipetting.

27 Return the aggregates to the same ultra-low-attachment plate and redistribute an approximately equal number of aggregates in each well with the p1000 pipette. Feed every other day until day 30.

Day 30: Selection and plating of OLIG-2-enriched aggregates (Timing 1–4 h)

28 Have a poly-L-ornithine and laminin 6-well plate ready to go.

Note

- (!) Use  1 mL of  50 µL poly-L-ornithine in dH2O per well of a 6-well plate and incubate at  37 °C  Overnight . Poly-L-ornithine coated plates can be stored at  37 °C for four days.
- After overnight incubation, aspirate poly-L-ornithine and allow plate to air dry for  00:05:00 . Add  1 mL of  20 µL natural mouse laminin in DMEM/F12 to each well.
- Incubate the plate for at least  04:00:00 at  37 °C . Poly-L-ornithine and laminin coated plates can be stored at  37 °C for two days.

29 Add  3 mL of PDGF medium per well.



- 30 Use a microscope under sterile conditions, and with a p200 pipette pick the aggregates that are round, that have a diameter of 300 - 800 μm , and that appear golden or brown with a dark center. Plate 20 spheres in a well of a six-well plate.

OPC differentiation in adherent cultures (Timing 35 d)

- 31 Every other day, carefully replenish two-thirds of the medium with fresh PDGF medium until day 65 of differentiation.
- 31.1 Be extremely gentle during medium changes. Do not tilt the plate to aspirate the old medium. The cell aggregates should be covered with liquid at all times.
- Gently aspirate the old medium with a p1000 pipette, by placing the tip close to the wall, without disturbing the cells.
 - Add fresh medium very slowly by aiming at the wall of the well.
 - Avoid rough and sudden movements even when you transfer the plate from the incubator, especially after day 40, because the cells will detach from the plate, typically in the form of a sheet.

Preparing Cells for MACS

48m

- 32 Remove media from cells and add with  1 mL of accutase per well of a 6-wellplate. Incubate for  00:20:00 at  37 °C .
- 33 Check under the scope to see if cells have lifted off plate. Incubate for another  00:05:00 -  00:10:00 at  37 °C if needed.
- 34 Dilute Accutase with  2 mL of DMEM with  10 micromolar (μM) Y27632 and place in a 15mL conical.
- 35 Spin down at  200 x g, Room temperature, 00:04:00 .
- 36 Aspirate media and resuspend in  4 mL of DMEM with  10 micromolar (μM) Y27632 and  100 μL DNase and incubate for  00:05:00 at  Room temperature .

20m



10m



4m



5m





37 Gentle pipette up and down with a P1000 to break up the clumps.



38 Incubate for an additional 00:05:00 .

5m



39 Gentle pipette up and down

40 Run cells through a cell strainer.

- Make sure to rinse strainer with DMEM right before placing cells on it.

41 Spin down at 200 x g, Room temperature, 00:04:00 .

4m



42 Aspirate media and resuspend in 60 μ L of cold 0.5% BSA.

Magnetic labeling

19m

43 Work fast, use pre-cooled solutions and keep cells cold, at all times. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

44 Mix the cell suspension well and incubate for 00:10:00 in the refrigerator.

10m



45 Add 40 μ L of the Anti-A2B5 MicroBeads per 10^7 total cells and incubate for 00:15:00 in the refrigerator.

15m



46 Wash cells by adding 2 mL of 0.5% BSA and spin down at 200 x g, Room temperature, 00:04:00 .

4m



47 Aspirate supernatant completely and resuspend in 1 mL of 0.5% BSA.



Magnetic separation

16h

- 48 Place column in the magnetic field of a suitable MACS® Separator.
- 49 Prepare column by rinsing with  500 μ L of 0.5% BSA.
- 50 Apply cell suspension onto the column.
- 51 Collect flow-through containing unlabeled cells in a 2mL or 5mL tube.
- 52 Wash column with  1 mL of 0.5% BSA 3 times. Collect unlabeled cells that pass through and combine with the flow-through from step 55. 
- 53 Remove column from the separator and place it on a suitable collection tube.
- 54 Pipette  1 mL of 0.5% BSA with  10 micromolar (μ M) Y27632 onto the column. Immediately flush out the magnetically labeled cells by firmly and SLOWLY pushing the plunger into the column. These are sorted OPCs. 
- 55 At this stage OPCs are ready for co-culture. Perform cell count. Please see the accompanying protocol section: "Day 0 of co-culture: Introducing OPCs to motor neurons"
- 56 Alternatively, to maintain OPCs, count cells and plate accordingly on poly-l-ornithine and laminin plates. Keep cells in  10 micromolar (μ M) Y27632 for  16:00:00 . 
Continue to maintain OPCs in PDGF media.

Note

(!) OPCs differentiate to astrocytes if they are maintained for more than 2 weeks in PDGF media so make sure to run relevant experiments soon after sorting.



- 57 Plate labeled and unlabeled cells and in imaging dish to check if any cells of interest remain.

Day -2: Replating hiPCS

9m

- 58 When the cells reach 70–90% confluency, remove the medium and add  1 mL of accutase.

- 59 Incubate the plate in a  37 °C incubator for  00:05:00 .

5m



- 60 Dilute the accutase solution by adding  2 mL of DMEM/F12 medium.

- 61 Use a cell lifter to remove cells from well and gently pipette the mixture 2–5 times with the p1000 pipette to fully dissociate the hiPCS cell colonies to single cells.

- 62 Transfer the cells in a conical tube.

- 63 Centrifuge the cells at  200 x g, Room temperature, 00:04:00 .

4m



- 64 Resuspend the cell pellet in  1 mL of E8 containing  10 micromolar (μM) Y27632 and count the cells with a hemocytometer.

- 65 Plate 7×10^5 cells per well on a Matrigel-coated six-well plate with E8 supplemented with  10 micromolar (μM) Y27632.

Day -1: Maintaining hiPCS

- 66 Aspirate and add fresh E8 media to remove Y27632.

Day 0: Neural induction



- 67 By now colonies should have grown evenly and reached 80-90% confluency. When cells have reached this point, proceed with differentiation.
- 68 Aspirate medium and add MNIM + SB4, LDN, RA, SAG to induce differentiation.
- 69 Change MNIM + SB4, LDN, RA, SAG daily until cells have reached day 5.

Day 6: Directed differentiation

- 70 Aspirate medium and add MNIM + SU, DAPT, RA, SAG to direct cells to motor neuron lineage.
- 71 Change MNIM + SU, DAPT, RA, SAG daily until cells have reached day 13.

Day 14: Motor neuron maintenance

- 72 At this stage, cells can be frozen for later use.
- 73 To replate cells, remove the medium and add  1 mL of accutase.
- 74 Incubate the plate in a  37 °C incubator for  00:10:00 . 
- 75 Dilute the accutase solution by adding  2 mL of DMEM/F12 medium.
- 76 Use a cell lifter to remove cells from well and gently pipette the mixture 2-5 times with the p1000 pipette to fully dissociate the motor neurons.
- 77 Transfer the cells in a conical tube.
- 78 Centrifuge the cells at  200 x g, Room temperature, 00:04:00 . 



- 79 Resuspend the cell pellet in  1 mL of MNMM containing  10 micromolar (μM) Y27632 and count the cells with a hemocytometer.
- 80 Plate 1×10^6 cells per well on a poly-l-ornithine and laminin six-well plate with MNMM supplemented with  10 micromolar (μM) Y27632.

Note

(!) Motor neurons have the tendency to detach from glass plates. To prevent this, use a P1000 to remove old replenish media. Do not use a vacuum aspirator.

Day 15: Motor neuron maintenance

- 81 Remove and add fresh MNMM to remove Y27632. Continue to feed cells with MNMM every two days until day 22.

Day 23: Replate motor neurons for co-culture

- 82 To replate cells, remove the medium and add  1 mL of accutase.
- 83 Incubate the plate in a  37 °C incubator for  00:10:00 . 
- 84 Dilute the accutase solution by adding  2 mL of DMEM/F12 medium.
- 85 Use a cell lifter to remove cells from well and gently pipette the mixture 2–5 times with the p1000 pipette to fully dissociate the motor neurons.
- 86 Transfer the cells in a conical tube.
- 87 Centrifuge the cells at  200 x g, Room temperature, 00:04:00 . 



- 88 Resuspend the cell pellet in  1 mL of MNMM containing  10 micromolar (μM) Y27632 and count the cells with a hemocytometer.
- 89 Plate 5×10^4 cells per well on a poly-L-ornithine and laminin 24-well glass bottom plate with MNMM supplemented with  10 micromolar (μM) Y27632.

Note

(!) Glass plates are used here to prepare cells for immunofluorescence staining and visualization experiments. Motor neurons have the tendency to detach and form aggregates if kept on glass for more than 20 days. If experiments other than immunofluorescence are intended, plastic plates can be used.

Day 24: Motor neuron maintenance

- 90 Remove and add fresh MNMM to remove Y27632.

Day 26: Introducing co-culture media to motor neurons

- 91 Remove and add fresh media mixture composed of 50% MNMM and 50% co-culture media. The following day, the cell will be ready for co-culture.

Day 0 of co-culture: Introducing OPCs to motor neurons

- 92 Use the OPC cell count obtained after sorting.
- 93 Prepare a cell solution in co-culture media with  10 micromolar (μM) Y27632 at a density of 1×10^5 cells per mL.

Note

(!) Y27632 is used here as OPCs were just sorted.



- 94 Remove media from motor neurons and replenish with cell suspension ( 500 μ L for a 24-well plate) from step 36.

Day 1 of co-culture: Maintaining co-culture

- 95 Remove and add fresh co-culture media to remove Y27632.
- 96 Continue to feed cells every other day with co-culture media until day 20 when they are ready for fixation.

Protocol references

Douvaras and Fossati, 2015 (PMID: 26134954)

Ziller and Ortega, et al., 2018 (PMID: 29551301)

García-León et al., 2018 (PMID: 29337119)