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We use this protocol and it's working

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iPSC to Motor Neuron Differentiation Various Protocols

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ABSTRACT

iPSC to Motor Neuron Differentiation Various Protocols

MATERIALS

Note

Aliquoting notes: **All aliquots should be 1 use, please don't refreeze any aliquoted reagent.**

- Ascorbic acid P212121 - StemCell Technologies (Catalog # 72132)
  -Need a [M] 200 millimolar (mM) stock: dilute 500 mg powder into 14.1965 mL nuclease free water. Aliquot into smaller quantities. Store in -20 °C .

- DMH1 - StemCell Technologies (Catalog # 73634)
  -Need a [M] 10 millimolar (mM) stock: dilute 10 mg powder into 2.6288 mL DMSO. Aliquot into smaller quantities. Store in -20 °C .

- SB-431542 - StemCell Technologies (Catalog # 72234)
  -Need a [M] 10 millimolar (mM) stock: dilute 10 mg powder into 2.6015 mL DMSO. Aliquot into smaller quantities. Store in -20 °C .

- CHIR99021 - StemCell Technologies (Catalog # 72054)
  -Need a [M] 3 millimolar (mM) stock: dilute 10 mg powder into 7.1638 mL DMSO. Aliquot into smaller quantities. Store in -20 °C .

- Purmorphamine- StemCell Technologies (Catalog # 72204)

  -Need a [M] 10 millimolar (mM) stock: dilute 5 mg powder into 0.9604 mL DMSO. Aliquot into smaller quantities. Store in -20 °C.

- Valproic Acid- StemCell Technologies (Catalog # 72292)

  -Need a [M] 1 M stock: dilute 500 mg powder into 3.008 mL nuclease free water. Aliquot into smaller quantities. Store in -20 °C.

  -(Note: Product sheet lists PBS, DMSO, abs EtOH for dilution, but we use nuclease free water)

- Retinoic Acid- Sigma (Catalog # R2625-50mg)

  -First, make a [M] 50 millimolar (mM) stock: dilute 50 mg powder in 3.33 mL DMSO.

  -Then from that [M] 50 millimolar (mM) stock, make a [M] 1 millimolar (mM) stock: Take 100 µL of the [M] 50 millimolar (mM) stock and mix it with 4.9 mL DMSO.

  -Now that you have the desired [M] 1 millimolar (mM) stock you need to use for the differentiation, aliquot it in smaller quantities. Store in -20 °C. Also, keep the leftover [M] 50 millimolar (mM) stock you have, so you can make more [M] 1 millimolar (mM) stock from it when you run out.

  -Possible Troubleshooting if there are problems: We have a good protocol with this method, but if future troubleshooting is needed, the manufacturer recommends chloroform as diluent, then storage in -80 °C. (Link to manufacturer recommendation: Sigma r2625)

- Compound E- StemCell Technologies (Catalog # 73952)

  -Need a [M] 1 millimolar (mM) stock: dilute 500ug powder in 1.0194 mL DMSO. Aliquot into smaller quantities. Store in -20 °C.

- ROCKi (Y-27632)- StemCell Technologies (Catalog # 72304)

  -Need a [M] 10 millimolar (mM) stock: dilute 5 mg of the powder in 1.56 mL nuclease free water. Aliquot into smaller quantities. Store in -20 °C.

  -(Product Note: Also able to dilute in PBS (pH 7.2) ≤ 30 mM. DMSO ≤ 90 mM. Absolute ethanol ≤ 15 mM. For example, to prepare a [M] 5 millimolar (mM)

stock solution in PBS or water, resuspend 1 mg in 624 µL of PBS (pH 7.2) or water.)

-ROCKi is not used in the NComm paper, but YiHsien and Vijay use this as part of their protocol to maintain single cell survival.

- N2 Supplement (100X)- Thermo Fisher (Catalog # 17502001)

-Already at correct stock concentration straight from the bottle. Aliquot into smaller quantities. Store in -20 °C.

- B27 Plus Supplement (50X)- Thermo Fisher (Catalog # A3582801)

-Already at correct stock concentration straight from the bottle. Aliquot into smaller quantities. Store in -20 °C.

- DMEM/F12 (1:1)- Thermo Fisher (Catalog # 11320033) store in 4 °C.

- Neurobasal Media- Thermo Fisher (Catalog # 21103049) store in 4 °C.

- StemPro Accutase- Thermo Fisher (Catalog # A1110501)
- -Store in 4 °C, use at 1X.

-NComm paper uses this at later steps, as well as YiHsien (but he allows either this or TrypLE). It is NOT currently used in the protocol. May be a reasonable substitute if TrypLE is on backorder - otherwise additional testing is needed. Another option listed in the paper is Dispase, but we do not use that in the protocol.

- GlutaMAX Supplement (100X)- Thermo Fisher (Catalog # 35050061)

-Keep at Room temperature

-Already at correct concentration.

- Penicillin/Streptomycin- Thermo Fisher (Catalog # 15140122)

-Keep at -20 °C, aliquot into smaller quantities.

- TrypLE Select (1X)- Thermo Fisher (Catalog # 12563011)

-Keep at Room temperature. Already at correct concentration if using 1X.

- Cryostor CS10- Stem Cell Technologies(Catalog # 07930)
  -Keep at 4 °C
  -Already at correct concentration.

- Mouse Laminin - Gibco (Catalog # 23017-015)
  -Comes at a stock concentration between [M] 0.5-2.0 mg/mL
  -Working concentration for coating 1:300 (dilute in Molec Grade H2O)
  -Aliquot in one time use tubes. Store initially at -80C, then aliquots should be stored in -20 °C (<6 months).
  -Note: Laminin should be treated very carefully (no more freeze thaws), thaw slowly

- Poly-D-Lysine (PDL) - Sigma (P0899 - [M] 100 mg/mL)
  -Working concentration for coating is 1:2500 (dilute in Molec Grade H2O) for rafts.

- OLD PROTOCOL: Poly-D-Lysine (PDL) - Thermo Fisher (Catalog # A3890401)
  -Purchased bottle comes at correct stock concentration ([M] 0.1 mg/mL).
  -Working concentration for coating is 1:2500 (dilute in Molec Grade H2O)
  -Store in -20 °C .

- Matrigel Matrix hESC qualified - Corning (REF: 354277)
  -Each lot number of matrigel has a specific dilution factor that needs to be looked up on the corning website. Refer to this iPSC Basic Maintenance Protocol for how to dilute/resuspend matrigel and coat vessels: https://docs.google.com/document/d/1SHsnDDBfpc1NrD6D8PG0zEAY68w7QMn0iZm8rL64Vvc/edit

- Insulin-like growth factor 1 (IGF-1) - StemCell Technologies (Catalog #: 78022, 78142) (We use Cat. # 78022. Cat. # 78142 is Animal component free)
  -Stock: 100ug dissolved in 1000ul molecular grade water
  -Store in -80 °C . Aliquot into smaller quantities.

- Brain-derived neurotrophic factor (BDNF) - Stem Cell Technologies (Catalog #: 78005)
  -Stock: 10ug dissolved in 100ul molecular grade water.

-Store in -80 °C . Aliquot into smaller quantities.

- Ciliary neurotrophic factor (CNTF) - Stem Cell Technologies (Catalog #: 78010)
  -Stock: 10ug dissolved in 100ul molecular grade water.
  -Store in -80 °C . Aliquot into smaller quantities.

- cAMP (cyclic AMP) - (getting exact specs)

Note

Filtering note: Andrew Yoo's lab filters certain media components. We do not usually do this. If filtering is desired, or contamination is suspected, then add all of the non-protein components to the base media, and before adding the proteins (growth factors, B27, B2, Serum for example), then sterile filter the bottle. Afterwards, add the additional components.

iPSC/MNP Passaging TrypLE Protocol

1 Aspirate media off

2 Rinse with DPBS-/-

3 Add 1X TrypLE, 1ml per 6 well, 2ml per T-25, 3ml per T-75 (can use as low as 0.75X TrypLE according to GESC, dilute in 0.5mM EDTA pH 8)

4 Put in incubator until you see cells detaching (2 minutes to 5 minutes MAX), might have to move flask around to aid in that process

5 When cells detach, take out of the incubator and add 2x the original volume minimum of fresh media in the vessel (to stop enzymatic reactions)

6 Take cells and put them in a 15ml conical tube

7 Spin down at 1200rpm for 5 min (200g/RCF)

8 Take off supernatant, and resuspend cell pellet in the media.

9 Media per well in 6-well is 2ml, 12-well is 1ml, T-25 is 5ml, T-75 is 20 mL

10 IMPORTANT: Add ROCKi once you replate cells. 1ul ROCKi per 1ml of media in the vessel. Remove ROCKi via media change after 24 hrs. (ROCKi is not in the NComm paper, but is suggested by YiHsien and Vijay since it is commonly used to promote survival after single-cell dissociation and is used in the protocol. We have tested with and without and found significantly better differentiation to MNs with ROCKi). Note: RevitaCell is another ROCKi inhibitor (may be better than Y-27632).

MNP freezing protocol (to be used during any step of differ...

11 Perform single cell dissociation (see above for TryplE protocol - no ROCKi) up until and including the spin down at 200g for 5 min.

12 Resuspend in 1 mL Media, do the cell count

13 Spin down again. Aspirate the supernatant, resuspend in 1mL of Cryostor CS10 (or as many ml as vials you want to freeze down, ex: 2ml resuspension = 2 vials frozen down)

14 Transfer the Cryostor/Cell solution with from the 15ml conical tube to the cryovial (1 ml per cryovial)

15 Label tube (list that it is MNP, list if it has a library/guide/or no library, list what passage iiPS it came from, list the Step and Day it will be when we thaw, write date, write initials, write estimated cell count)

16 Place vials on ice for 10 minutes before putting it in a styrofoam box in the -80C Freezer.

17 After 24-48h transfer to permanent storage box in -80C.

PDL/Laminin Coating Procedure

18 PDL Instructions

18.1 Prepare a 1:2500 dilution of PDL in molecular grade water, making sure you have enough to coat whatever vessel you are working with (For a quad array: dissolve 2uL of PDL in 5mL of molecular grade water.)

18.2 Add 1mL per quad on the array.

18.3 Incubate at 37 degrees for 1 hour.

18.4 After 1 hour incubation, rinse thrice with molecular grade water as PDL can be toxic to cells.

19 Mouse Laminin Instructions

19.1 Thaw laminin at 2-8C to prevent it from gelling

19.2 Prepare a 1:300 dilution of laminin in molecular grade water, making sure you have enough to coat whatever vessel you are working with (For a quad array: Dissolve 14uL of laminin in 4.2mL of molecular grade water and add 1mL of the solution per quad)

19.3 Incubate at 37 degrees for 4 hours.

19.4 Aspirate laminin before use. Coated vessels can be used immediately without washing.

Note

In Yi-Hsien's protocol, he mentions after PDL coating has been rinsed off 3 times, to leave the cultured vessel uncovered in the hood to dry for 2 hours- we do not do this currently, we just add the laminin coating right away. He also mentions that laminin only needs to be incubated for 2 hours at 37C or overnight at 2-8C, we currently incubate it for 4 hrs at 37C.

Motor Neuron Differentiation Protocol

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Note

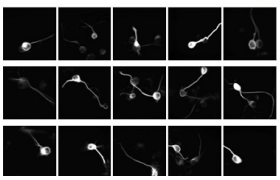
Original source: https://www.nature.com/articles/ncomms7626

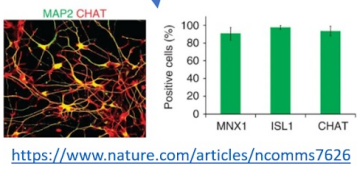
Components	Stock Conc.	Step 1: Neuroepithelial induction (Sox1+)	Final conc.	Step 2: Olig2+/NKX2 - MNP	Final conc.	Step 3: MNP expansion	Final conc.	Step 4: Induce MNX1+ MN	Final conc.	Step 5: Maturation of CHAT+ MN ISL1,MAP2+)	Final conc.	Step 6: Long Term Culture	Final conc.
Pen Strep (antibiotic)	100X	Pen Strep	1X	Pen Strep	1X	Pen Strep	1X	Pen Strep	1X				
DMH1 (dorsomorphin = BMP inhib)	10mM	DMH1	2 uM	DMH1	2 uM	DMH1	2 uM						
SB-431542 (Activin/Nodal inhib)	10mM	SB-431542	2 uM	SB-431542	2 uM	SB-431542	2 uM						
CHIR99021 (GSK3 inhib)	3mM	CHIR99021	3 uM	CHIR99021	1 uM	CHIR99021	3 uM						
Purmorphamine (shh activator)	10mM			Purmorphamine	0.5 uM	Purmorphamine	0.5 uM	Purmorphamine	0.1uM	Purmorphamine	0.1uM		
Retinoic acid (promotes diff)	1mM			Retinoic acid	0.1 uM	Retinoic acid	0.1 uM	Retinoic acid	0.5 uM	Retinoic acid	0.5 uM	Retinoic acid	1 uM
Valproic acid (HDAC inhib)	1M					Valproic acid	0.5 mM						
Compound E (Notch inhibitor)	1mM									Compound E	0.1 uM		
cAMP (2nd Msg. Survival)												cAMP	0.1 uM
IGF/BDNF/CNTF (Growth Factors)												IGF/BDNF/CNTF	10ng/ml
	Days:	6		6		Varies		6		10		Varies	
	Substrate	Matrigel		Matrigel		Matrigel		Matrigel		PDL/Laminin		PDL/Laminin	

Base Media:

DMEM/F12	1X	50%
Neural basal medium	1X	50%
N2	100X	0.5X
B27	50X	0.5X
Ascorbic Acid	200 mM	0.1 mM
GlutaMAX	100X	1X

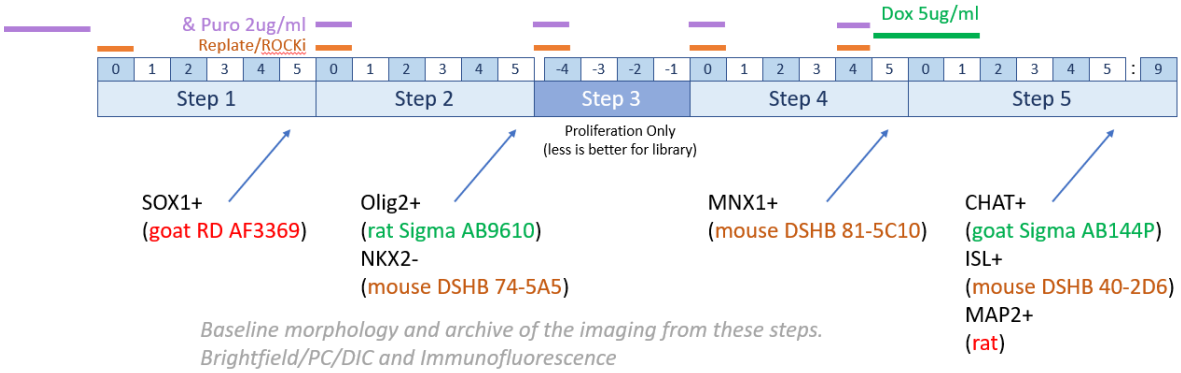
"IGF1, BDNF, CNTF were added if MNs were plated at low density. For identifying MN disease phenotypes (ALS), cultured **without** these factors"





https://www.nature.com/articles/ncomms7626

iPSC Protocol Slides - Google Slides



Baseline morphology and archive of the imaging from these steps. Brightfield/PC/DIC and Immunofluorescence

Note

How to Count Days on Step Media
When you place cells into a new Step Media, label this as Day 0. The next day is Day 1 and so on. Late in Day 5 or early in Day 6, switch to next media and again label cells as Day 0 of the next step.

When freezing a tube, freeze it at the end of the day it is on and write the next day on the label of the frozen tube. **So, when thawing a tube, write the day written on the vial on the flask/plate you are putting it into.**

Protocol Overview
To differentiate the iPSC cells, you have to grow them in 5 or 6 different media.
From this point forward, only use the TrypLE protocol above when passaging. As stated in that protocol, add ROCKi EVERY TIME you use TrypLE (1ul/ml media). Remove ROCKi after 24hr via media change unless you are in the later stages of differentiation (Step 4 or Step 5- media changes are less frequent).
To start the differentiation process, transfer an appropriate amount of cells into your desired vessel using the TrypLE protocol listed above to single-cell dissociate iiPS cells. Use STEP1 media to resuspend and store the cells in a new matrigel coated vessel.

Note

Additional Notes:

- See iPSC protocol to start initial iPSC culture and get matrigel information https://docs.google.com/document/d/1SHsnDDBfpc1NrD6D8PG0zEAY68w7QMn0iZm8rL64Vvc/edit
- Keep each step media fresh- use within 4 days of making it.
- For non-library differentiation
- Use 1 full well of a 6well plate (from previous protocol) into 3 T-25 as a starting point (around a few million cells total)
- Try to keep confluency around 80%
- For library differentiation
- Make sure that at all times, you have at least 1000X coverage (number of cells= 1000 * # gRNAs in the library). Write on flask: “at all times maintain a minimum XXXX cell number”. Maintain this number when passaging. If the cell number is too large, it can be split into multiple vessels, but keep this in mind when freezing down, so you can combine cells which are in multiple vessels to maintain good library representation.
- At each step, even during the step, passage using the TryplE protocol as soon as cells get to 90%-95% confluency. Being crowded is fine, but do not let cells get overcrowded.
- We use 0.1% P/S instead the original 1% in Yi-Hsien’s protocol, because we found that to have better cell survival and we can pick up on contamination faster (Waleed also uses this concentration)
- **If making a library, please freeze at every step just in case future steps go poorly.**

Components added to the base media at each step have been bolded.

21 Step 1 Media Recipe

Step 1 (6 days) NEP (neuroepithelial cells) induction	Stock Conc.	Final conc.	10 ml dilution	50 ml dilution
DMEM/F12		50%	4.86ml	24.3ml
Neural basal medium		50%	4.86ml	24.3ml
N2	100X	0.5X	50ul	250ul
B27	50X	0.5X	100ul	500ul
Ascorbic acid	200 mM	0.1 mM	5ul	25ul
GlutaMAX	100X	1X	100ul	500ul
P/S .1%	100X	1X	10ul	50ul
DMH1	10mM (5000x)	2 uM	2 ul	10ul
SB-431542	10mM (5000x)	2 uM	2 ul	10ul

CHIR99021	3mM (1000x)	3uM	10 ul	50ul

Note

Note: In the original paper, they use lower density cultures and therefore do media changes less frequently. Since we are working with more cells/ need to differentiate more cells, we have optimized for higher density, and therefore need more frequent media changes at lower Steps.

Leave the STEP1 media on the cells for 6 days, doing media changes everyday. Over the weekend, if you need to and the well is not too crowded, you can double feed the cells and let them go for 2 days. On the last days of STEP1 media, you may start seeing swirls in the clusters of cells (unless density is high).

If you need to split during this time, use the TrypLE protocol (adding the ROCKi) then re-plate in STEP 1 media. Do a media change after 24 hrs to remove ROCKi.

By the end of STEP1, the iPSCs are SOX1+ NEP (neuroepithelial progenitor cells) or the earliest multipotent neural stem cells. You can freeze the NEP during this stage if you want to store them.

On the end of “Day 5” or beginning of “Day 6”, single-cell dissociate using TrypLE and ROCKi, and re-plate in STEP2 media. Cells should be re-plated in a new matrigel-coated flask/well. Do a media change after 24hr to remove the ROCKi.

22 Step 2 Media Recipe

Step 2 (6 days) Induce Oligo2+ MNP	Stock Conc.	Final conc.	10ml dilution	50ml dilution
DMEM/F12		50%	4.86ml	24.3ml
Neural basal medium		50%	4.86ml	24.3ml
N2	100x	0.5X	50ul	250ul
B27	50x	0.5X	100ul	500ul
Ascorbic acid	200 mM	0.1 mM	5ul	25ul
GlutaMAX	100X	1X	100ul	500ul
P/S 0.1%	100X	1X	10ul	50ul
DMH1	10mM (5000x)	2 uM	2 ul	10ul
SB-431542	10mM (5000x)	2 uM	2 ul	10ul
CHIR99021	3mM (3000x)	1uM	3.3 ul	16.5ul

Pur	10mM (20000x)	0.5uM	0.5 ul	2.5ul
Retinoic acid	1mM (1000x)	0.1 uM	1 ul	5ul

Do a media change everyday or double feed if you cannot come in over the weekend. At the end of this step, the cells are Olig2+/NKX2- Motor Neuron Progenitors (MNPs).

If you need to split during this time, use the TrypLE protocol (adding the ROCKi) then re-plate in STEP 2 media. Do a media change after 24 hrs to remove ROCKi.

At the end of Day 5 or beginning of Day6, if the cell number is high enough, you can go straight into Step 4. Alternatively, you can proliferate the MNPs in Step 3 media to generate higher cell numbers. Passage with TrypLE/ROCKi after Step2 and re-plate in Step 3 media into matrigel coated flasks/wells. Do a media change after 24hr to remove ROCKi.

23 Step 3 (MNP expansion) Media Recipe

Step 3 (MNP Expansion)	Stock Conc.	Final conc.	10ml dilution	50ml dilution
DMEM/F12		50%	4.86 ml	24.3ml
Neural basal medium		50%	4.86 ml	24.3ml
N2	100X	0.5X	50ul	250ul
B27	50X	0.5X	100ul	500ul
Ascorbic acid	200 mM	0.1 mM	5ul	25ul
GlutaMAX	100X	1X	100	500ul
P/S 0.1%		1X	10ul	50ul
DMH1	10mM (5000x)	2 uM	2 ul	10ul
SB-431542	10mM (5000x)	2 uM	2 ul	10ul
CHIR99021	3mM (1000x)	3uM	10 ul	50ul
Pur	10 mM (20000x)	0.5uM	0.5 ul	2.5ul
VPA (valproic acid)	1 M (2000x)	0.5mM	5 ul	25ul
Retinoic acid	1mM (1000x)	0.1 uM	1 ul	5ul

The cells can be left in this media for a MAXIMUM of **5** days (the smallest amount of time in step 3 is ideal, so we jackpot less). Media change daily or double feed over the weekend. After this step, the cells will be motor neuron progenitors. At this point, we can expand and freeze many vials down.

After this step, the cells should be single cell dissociated using TrypLE/ROCKi and moved to Step 4 media in a new matrigel flask/well. Remove ROCKi after 24 hrs.

24 Step 4 Media Recipe

	Step 4 (6 days) – induce MNX1+ MNs	Stock Conc.	Final conc	10 ml dilution	50 ml dilution
	DMEM/F12		50%	4.86 ml	24.3ml
	Neural basal medium		50%	4.86 ml	24.3ml
	N2		0.5X	50ul	250ul
	B27		0.5X	100ul	500ul
	Ascorbic acid	200 mM	0.1 mM	5ul	25ul
	GlutaMAX		1X	100ul	500ul
	P/S 0.1%		1X	10ul	50ul
	Pur	10 mM (100000x)	0.1uM	0.1 ul	0.5ul
	Retinoic acid	1mM (1000x)	0.5 uM	5 ul	25ul

While the cells are on STEP 4 media, they will mature into MNX1+ motor neurons. Reduce media changes to full media changes every other day.

At the end of Day 5 or beginning of Day 6, single-cell dissociate the cells with TrypLE/ROCKi and move to a PDL/Laminin coated vessel in Step 5 media.

Note

Note: FIVE@MGI normally freezes at Step 4 Day 3, but we are optimizing currently.

25 Step 5 Media Recipe

	Step 5 (10days) – maturation into CHAT+ MN	Stock Conc.	Final conc	10 ml dilution	50ml dilution
	DMEM/F12			4.83ml	24.3ml
	Neural basal medium			4.82ml	24.3ml
	N2			50ul	250ul
	B27			100ul	500ul
	Ascorbic acid	200 mM		5ul	25ul
	GlutaMAX			100ul	500ul
	Pur	10 mM (100,000x)	0.1uM	0.1 ul	0.5ul
	Retinoic acid	1mM (1000x)	0.5 uM	5 ul	25ul
	Compound E	1mM (10,000x)	0.1 uM	1 ul	5ul

At step 5, perform half medium change every other day. **No full medium change at this step.**

Note

Note: FIVE@MGI is performing ongoing experiments to determine how much longer we can go in Step 5 before being able to replate.

At high density, and early days of Step 5, growth factors are not required. For plating densities on Raft Arrays, lower densities, or longer times, Step 6 media (which includes cAMP and Growth Factors) should be used to maintain neuron survival. For studying neurodegenerative models, the paper removes the neurotrophic support.

For long term motor neuron growth, do a media change on late stage Step 5 cells and put them on Step 6 media. Yi Hsien had noted that the best time to move neurons was around Step 5 Day 4 or Day 5.

26 **Step 6 Media Recipe**

Step 6 (Long term culture)	Stock Conc.	Final Conc.	10 ml dilution	50 ml dilution
Neurobasal medium			9867ul	49,335ul
N2		1X	100ul	500ul
cAMP	50uM	0.1 uM	20ul	100ul
ILGF/BDNF/CNTF	100ng/ul	10ng/ml each	1ul each	5ul each
Retinoic acid	1mM (1000x)	1 uM	10ul	50ul

Note

Additional notes from Andrew Yoo's group:
https://www.cell.com/cell-stem-cell/fulltext/S1934-5909(17)30320-X
(Note that these are for Direct Differentiation and therefore aren't immediately applicable)

- Drop wise plating technique
- Differentiation from fibroblasts to neurons: they use a hybrid media cocktail inline with our step 5 and 6 recipes with a couple different additives:
- Neuronal Media (ScienCell, 1521) supplemented with Dox
- valproic acid (1 mM; EMD Millipore)
- dibutyryl cAMP (200 µM; Sigma-Aldrich)
- Retinoic Acid (1 uM; Sigma-Aldrich)
- Growth Factors
- BDNF
- **NT-3**
- CNTF
- **GDNF**
- Neuronal media, cAMP are in our step 6 media. Retinoic acid is in our step 5 media.. NT-3 and GDNF are different growth factors than what we use.
- Dox replenished every 2 days.
- Half-media changes every 4 days.

Diagram of the protocol is found in S1:

https://www.cell.com/cms/10.1016/j.stem.2017.08.002/attachment/f0f514a9-2cb1-4f44-9bb2-f736fa9c1f8a/mmc1