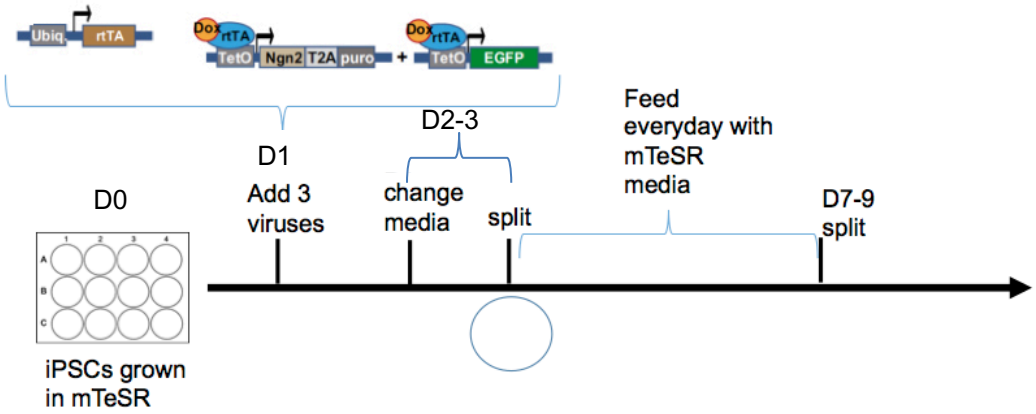
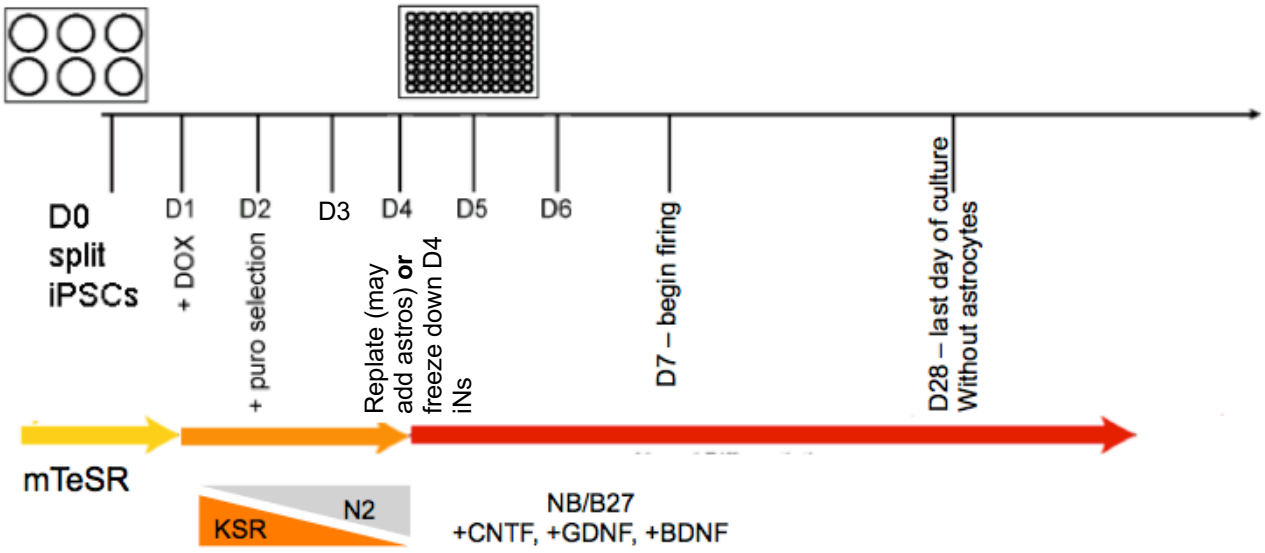
Protocol for differentiation of excitatory cortical projection neurons from transduced human ES and iPS cells with Neurogenin 2 (NGN2) virus

Overview and protocol (Based upon Zheng…Sudhof 2013, modified by Eggan lab, modified by TYP lab):

Making NGN2 inducible neuron lines from iPSCs:



Differentiating NGN2s to inducible neurons (iNs):



Viral Transduction of iPS Cells with -TetO-Ngn2- Puro, TetO-GFP, Ubq-rtTA to make NGN2 inducible neuron iPSCs:

When doing viral infections, be sure to wear sleeves to avoid contamination; dispose of tips, tubes, etc. in bleach; and wipe down surface used with bleach.

Day 0

1. Plate iPS cells in 1 well of 12 well plate with 380,000 cells per well (**~100,000 cells/cm^2**) in mTeSR media + ROCKi (10 uM). Plates are coated with growth-factor-reduced Matrigel (**Matrigel-GFR #354230 from Corning**)**.** See attached Matrigel protocol for more info.
    - 10 uM ROCKi is 1:1000 dilution of 10 mM stock

Day 1

1. Collect lentivirus from -80C freezer and keep on ice.

2. Make-up master mix with following concentrations:
   Per well of 12wp, in 1 mL of **mTeSR media** (concentrations based on our ultrapure titre >10^9):

	Titre	concentration	uL per 12 well (380,000 cells)
pTet-O-NGN2-puro lentivrirus	**2.97x10^9**	0.132 uL/ 50K cells	1.0
Tet-O-FUW-eGFP lentivirus	**2.05x10^9**	0.05 uL/ 50K cells	0.4
Fudelta GW-rtTA lentivirus	**1.34x10^9**	0.132 uL/50K cells	1.0
CAMKII lentivirus	**1.78x10^9**	0.05 uL/ 50K cells	0.4

NOTES:

* GFP and CAMKII are optional. Titres listed above are from a recent batch of NeuroHub virus. **Make sure to edit the uLs to use based on your virus titre.**

** For NGN2 and rtTA virus - concentrations of 0.11 uL/50K cells have also been used, depending on the batch titre.

*** *NeuroHub recommends using mTeSR for culture of cells prior to and during virus transduction. There is increased cell death and reduced transduction efficiency with StemFlex media. StemFlex can be used during the passage after transduction.*

Day 2 (or 3)

- When confluent, split each well of 12wp to 1x10cm plate (almost always on **D2**).

  NOTE: Here cells can be switched to **StemFlex** and fed on the 6-7 day flex feeding schedule (see manufacturer's directions). Alternatively, **mTeSR** feeding every day can be continued.

Days 3-7

Feed with **StemFlex** (flex schedule) or **mTeSR** (every day). When cells look confluent in 10cm plate (usually around **D6** or **D7**), split for maintenance/expansion. **Count cells!** Usually should expand 1→5-10 (plating **2E6 cells/10cm plate**). See splitting protocol for more info (Splitting iPSCs on Matrigel/mTeSR, Part IIB).

NOTES:

- Expand and freeze down cells **after transducing but before expanding/banking D4 neurons**!!!
  - You want to have a stock of already transduced cells so that you do not have to go through the time and expense of a second round.
- Plate **2E6 cells/10cm plate** for maintenance/expansion.
- Perform a test differentiation to D4 using a small number of wells in a 96wp.
- Freeze down cells at earlier passage #; NGN2 lines do not differentiate well past **P10** after virus.

Protocol for generating iNs (induced neurons) from NGN2s (iPSCs transduced with virus):

Step 1: Maintain NGN2 cells at **~2E6 cells/10cm plate** (**~100,000 cells/cm^2**)

- Feed 10cm plates with 7 mL per media plate
    - If using **mTeSR**, feed *everyday* and split every ~7 days
    - If using **StemFlex**, feed and split according to flex schedule

Step 2 (D0): For differentiation, split cells and plate **~4E6 cells/10cm plate** (**200,000 cells/cm^2**) using mTeSR (or StemFlex) media + ROCKi (10 uM)

- 10 uM **ROCKi is 1:1000** dilution of 10 mM stock
- *If only keeping cells for maintenance, plate **2E6 cells/10cm plate** and continue to feed regularly*
- *You may want to make sure D0 falls on a Thurs. or Fri. so that D4 never ends up on a weekend!*
- ***Do not proceed to Step 3 (D1) until cells in 10cm plate are at least ~75% confluent. Maintain in mTeSR or SF media (hold at "D0").***

Step 3 (D1): When cells are at least ~75% confluent, feed with **KSR media + doxycycline (dox; 2 ug/mL)** to induce Ngn2 expression (and GFP if present)

- 2 ug/mL **dox is 1:10k** dilution of 2 mg/mL stock
- The addition of factors **SB/XAV/LDN** has been shown to improve neuron maturity (Nehme, Cell Reports, 2018) – see below:

Inhibitor	Vendor	Cat no.	[Stock]	[Final]	Dilution
LDN-193189	Stemgent	04-0074	500 uM	100 nM	1:5,000 DMSO
SB431542	Tocris	1614	10 mM	10 uM	1:1,000 EtOH
XAV939	Stemgent	04-00046	10 mM	2 uM	1:5,000 DMSO

Step 4 (D2): Feed with **1:1 ratio of KSR:N2B media + puromycin (puro; 5 ug/mL) + dox (2 ug/mL)** to select for transduced cells

- 5 ug/mL **puro is 1:2000** dilution of 10 mg/mL stock
- 2 ug/mL **dox is 1:10k** dilution of 2 mg/mL stock
- ***NOTE**: you may need to optimize puro concentration depending on the cell line. We have tested within the **range of 1 ug/mL to 10 ug/mL**.*
- Again, **SB/XAV/LDN** can be added to the KSR media portion (so half of total volume of media).
- *All cells not expressing Ngn2/puro resistance gene should die off at this point*

Step 5 (D3): Feed with **N2B media + B27 (1:100) + puro (5 ug/mL) + dox (2 ug/mL)**. No more additional factors SB/XAV/LDN.

- 5 ug/mL **puro is 1:2000** dilution of 10 mg/mL stock
- 2 ug/mL **dox is 1:10k** dilution of 2 mg/mL stock

Step 6 (D4): Freeze D4 neurons (put in -80 for 1 night then transfer to LN2)

- Wash 10cm plate with PBS (3-4 mL) and add 3-4 mL diluted accutase (Gibco, A11105) solution per plate
    - 1:3 accutase:PBS + ROCKi (10 uM) (=1:1000 of 10 mM stock)
- Incubate cells for 3-5 minutes, or until cells have lifted off the place. Collect, count and spin down cells.
- Count cells! Freeze down 1-2E6 cells per cryovial with 1:1 ratio media:freezing media
    - **D4 iN media**: **NBM + B27 (1:50) + BDNF/CNTF/GDNF (all 10 ng/uL) + ROCKi (10 uM) + puro (5 ug/mL) + dox (2 ug/mL)**
        - **Note – B/C/G GFs are not necessary in the media for freezing aliquots**

- 10 ng/uL B/C/G is 1:1000 dilution of 10 ug/mL stock
- 10 uM ROCKi is 1:1000 dilution of 10 mM stock
- 5 ug/mL puro is 1:2000 dilution of 10 mg/mL stock
- 2 ug/mL dox is 1:10k dilution of 2 mg/mL stock

- **Freezing media**: 20% DMSO in FBS (e.g. 2 mL DMSO in 8 mL FBS; store at 4°C)
  - Cells should be in no more than 500 uL total volume per cryovial

Important notes:

- Accutase should never be warmed up at 37°C; this will inactivate it!
- If co-culture with glia is desired, add glia on D4 or D5 at ~70,000 cells/cm^2
  - *Usually if you are looking to study synaptic expression or e-phys/MEAs*
- As noted above, further optimization of puromycin on D4 and beyond may be needed depending on the line

Media Reagent Product Numbers:

Reagent	Vendor	Cat no.	[Stock]	[Final]	Dilution
BDNF	Peprotech	450-02	10 ug/mL in 0.1% BSA/PBS	10 ng/mL	1:1000
CNTF	Peprotech	450-13	10 ug/mL in 0.1% BSA/PBS	10 ng/mL	1:1000
GDNF	Peprotech	450-10	10 ug/mL in 0.1% BSA/PBS	10 ng/mL	1:1000
Rocki (Y-27632)	Stemcell Technologies	72304	10 mM solution	10 uM	1:1000

Other Reagents:

Reagent	Vendor	Cat no.	[Stock]	Dilution
B27	Life technologies	17504-044	10 mL	1:50 or 1:100
PBS	Invitrogen	14190-250	500 mL	
Puromycin	Life Technologies	A11138-03	10 mg/ml	1ug/mL- 10ug/mL (each line requires optimization)
DNASE1	Biolabs	MO30L	2000 uL	

Media Reagent Preparation Notes:

- **BDNF/CNTF/GDNF:**
  - Centrifuge vial prior to opening, 5 min at max speed, 4°C
  - Dilute 100 ug stock vial in 10 mL 0.1% BSA/PBS → pipette to mix
  - Aliquot working stock (now 10 ug/mL) in 500 ul volumes and store at -20°C
  - Use at 1000x (10 ng/mL)
- **ROCKi (Y-27632):**
  - Dilute 10 mg stock vial in DMSO (volume varies depending on MW of batch)
  - Aliquot working stock (now 10 mM) in 300 uL volumes and store at -20°C
  - Use at 1000x (10 uM)
- **Puromycin:**
  - Stocks are 1 mL aliquots at 10 mg/mL in HEPES; store at -20°C
  - Use at 10,000x-1000x (1-10 ug/mL)
- **Doxycycline hyclate** (Sigma D9891-5g, stored 4°C):
  - Weigh out 100 mg and dilute in 5 mL sterile water → pipette to mix
  - Aliquot working stock (now 20 mg/mL) in 300 uL volumes and store at -20°C
  - Use at 10,000x (2 ug/mL)

- **20% Dextrose** (Sigma D9434-250g, D-(+)-glucose, stored RT):
    - Weigh out 20 g and dilute in 100 mL MilliQ water → stir
    - Sterile filter and store at 4°C
- **N2 supplement B** (Stemcell Technologies 07156):
    - Store at -20°C and use at 100x

Media recipes

mTeSR media (1 L: 2x500mL kits)

- 800 mL mTeSR (StemCell Technologies, 05857)
- 200 mL mTeSR supplement
- 10 mL PenStrep
- *Sterile filter and store in 4°C*

Human KSR media (500 mL)

- 415 mL KO DMEM (Gibco)
- 75 mL Knockout Replacement Serum (KSR Invitrogen 10828-028)
- 5 mL MEM NEAA (Invitrogen, 11140-050)
- 0.5 mL beta-mercaptoethanol (Invitrogen, 21985-023)
- 5 mL Glutamax (Gibco, 35050)
- *Sterile filter, cover in aluminum foil, and store in 4°C*

N2B media (500 mL)

- 500 mL DMEM/F-12 (Life Technologies, 11330057)
- 5 mL Glutamax (Gibco, 35050)
- 7.5 mL 20% Dextrose
- 5 mL N2 supplement B *(add after filtration step)* (Stemcell Technologies 07156)
- *Sterile filter, cover in aluminum foil, and store in 4°C*

NBM (500 mL)

- 485 mL Neurobasal Medium (NBM) (Gibco, 21103)
- 5 mL Glutamax (Gibco, 35050)
- 7.5 mL 20% Dextrose
- 2.5 mL MEM NEAA (Invitrogen, 11140-050)
- *Sterile filter and store in 4°C*

Day 4 iN Plating Protocol from Thaw

- For best coating results: if using Poly-O-Laminin, coat night before. If using Matrigel, coat the day of plating and incubate for a least 1 hr.
- Use Matrigel #354234 – Corning – NOT GF-reduced!!!
- See attached Matrigel protocol for more information
- Warm up re-suspension media: **NBM + 1:100 B27 + 1:1000 ROCKi**
  - Media for thawing and washing from LN2 does not need GFs
- Warm up D4 plating media: **NBM + 1:50 B27 + 1:1000 ROCKi+ 1:1000 BDNF/ GDNF/CNTF + 1:2000 puromycin + 1:10K dox**
  - *Plating media requires GFs*
  - *If plating MEAs or co-cultures, omit the puro!*

1. Take cells from liquid nitrogen and immediately add to bead or water bath.
2. Take vial out of bath once they are 'just-thawed' (only about 2 minutes max).
3. Recover cells, adding **1 mL NBM + 1:100 B27 + 1:1000 ROCKi** per vial

   NOTE: always add media drop-wise when collecting cells in DMSO

   1. Transfer to 1 x 15 ml tube
   2. Wash cryotube with 1 mL media to collect any residual cells
   3. Add another 2-3 mLs DROPWISE of **NBM + 1:100 B27 + 1:1000 ROCKi**
   4. Triturate and then **take 15 uL of cell suspension from tube to count**

      % viability after thawing D4 neurons should be at least 80%.
4. Count/spin down: 200 g for 5 min.
5. Re-suspend cells in D4 plating media (**NBM + 1:50 B27 + 1:1000 ROCKi+ 1:1000 BDNF/ GDNF/CNTF + 1:2000 puromycin + 1:10K dox)**
6. Usually plate at 12.5-25K density in 96wp, see chart below to plan accordingly:

The chart lists cell number values to scale up from a 96wp:

Well #	Size (cm2)	15K cells/cm2	37.5K cells/cm2	78K cells/cm2
96	**0.32**	5.00E+03	1.20E+04	2.50E+04
48	**0.95**	1.48E+04	3.56E+04	7.42E+04
24	**1.9**	2.96E+04	7.12E+04	1.48E+05
12	**3.8**	5.93E+04	1.42E+05	2.96 E+05
6	**9.5**	1.48E+05	3.56E+05	7.42E+05

For incucyte: plate 5K cells/96well

For RNA seq: plate cells at 50K cells/96 well → usually 3.35e5 in a 24wp

For proteomics: plate cells at 1E6 in 1 well of a 6wp

7. D5: Perform a full media change media using **NBM + 1:50 B27 + 1:1000 BDNF/ GDNF/CNTF + 1:2000 puromycin + 1:10K dox)**.

8. D6-D21: Feed every 3-4 days (Mon/Thurs or Tues/Fri) with a 1/2 or 2/3 media change using **NBM + 1:50 B27 + 1:1000 BDNF/ GDNF/CNTF + 1:2000 puromycin + 1:10K dox)**.

- *Example - For 96wp with 200uL volume, pipette off 100uL and add 100uL new media.*
- *For 96wp with 100uL volume, pipette off 50uL and add 100uL new media.*

- *NeuroHub recommends discontinuing the use of doxycycline and puromycin between D7-10. This may need to be optimized depending on the cell line.*
- *NeuroHub recommends harvesting iNs by D21.*

Notes:

- To help mitigate cells detaching or pulling back from wells *feed gently with partial media changes* and do not touch tip to the bottom of the well.
- Prior to collecting media for analysis, perform a full media change on one media-change time-point before ideal collection.
    - *Example – If you plan to harvest cells on Friday (D21), perform a full-media change on Wed. D19 for 2 day conditioned-media.*

Plating MEAs

First- thaw rodent astrocytes into 1xT75 flask (cells located box E6) – see plating instructions protocol

- Coat MEA plates with poly-o-laminin (POL) for at least 3 hours (usually do this the night before)
- The next day, remove the POL and add 100 uL Matrigel to each well.
    a. **NOTE**: Matrigel is Corning cat #354234; we usually aliquot Matrigel into 0.5 mg aliquots (based on protein concentration of vial), resuspend in 6 mLs of cold, filtered DMEM/F12 and add 5 uL per well of MEA plate.
- Let Matrigel incubate for 1 hour.
- When you are ready to plate cells, remove Matrigel and wash once with PBS.
- We plate cells in a very low volume of media (for a 48wp - plate neurons in 30 uL of media)
- Usually plate iNs first and then astrocytes immediately after in another 30 uL of media. Attached are our plating densities.
    a. **NOTE**: Recently we have tried plating at an even lower density and may think that lower density is better.
    b. For a 48wp, plate 24K iNs, 24K primary rodent astrocytes
- After 3-4 hours (after cells have attached) flood wells with media and bring it up to 100 uL per well of 96wp or additional for larger wells.
    a. **NOTE**: There can be contamination issues if there is too much media because of water vapor on top of lid)
- Feed with NBM for the first 2 weeks (without dox and puro) and then switch over to Brain Phys (StemCell Technologies)
    a. BrainPhys + 1x50x supplement of SM1 (do not need to add any more growth factors)
    b. **NOTE**: You could probably feed with BrainPhys from the beginning but we wait a little because it is cheaper.