Astrocyte Differentiation (Sox9 & NfiB induction) - Adapted from Canals et al 2018) DRJ protocol

Astrocyte Expansion Medium (no more than 2 weeks)

Reagent	Initial [c]	For 500 mL	For 250 mL	For 125 mL	Final [c]
DMEM/F12, hepes		(RM 60 mL)	220 mL	99 mL	
FBS	100%	50 mL	25 mL	12.5 mL	10%
N2 Supplement	100x	5 mL	2.5 mL	1.25 mL	1% (1x)
Glutamax	100x	5 mL	2.5 mL	1.25 mL	1% (1x)

Astrocyte FGF Medium (no more than 2 weeks)

Reagent	Initial [c]	For 500 mL	For 250 mL	For 125 mL	Final [c]
Neurobasal		(RM 26 mL)	238 mL	119 mL	
B27 Supp (not B27-RA)	50x	10 mL	5 mL	2.5 mL	2% (1x)
NEAA	100x	5 mL	2.5 mL	1.25 mL	1% (1x)
Glutamax	100x	5 mL	2.5 mL	1.25 mL	1% (1x)
FBS	100%	5 mL	2.5 mL	1.25 mL	1%
bFGF (FGF2)	20 ug/mL	200 uL	100 uL	50 uL	8 ng/mL
CNTF	10 ug/mL	250 uL	125 uL	62.5 uL	5 ng/mL
BMP4	10 ug/mL	500 uL	250 uL	125 uL	10 ng/mL

Astrocyte Maturation Medium (no more than 2 weeks)

Reagent	Initial [c]	For 500 mL	For 250 mL	For 50 mL	Final [c]
DMEM/F12, Hepes Neurobasal (1:1 mixture)		242 mL 242 mL	121 mL 121 mL	23.5 mL 23.5 mL	
N2 Supplement	100x	5 mL	2.5 mL	500 uL	1% (1x)
Sodium Pyruvate	100 mM	5 mL	2.5 mL	500 uL	1% (1mM)
Glutamax	100x	5 mL	2.5 mL	500 uL	1% (1x)
N-acetyl-cysteine (NAC)	50 mg/mL	50 uL	25 uL	5 uL	5 ug/mL
Heparin-binding EGF-like growth factor (EGF)	50 ug/mL	50 uL	25 uL	5 uL	5 ng/mL
CNTF	10 ug/mL	500 uL	250 uL	50 uL	10 ng/mL
BMP4	10 ug/mL	500 uL	250 uL	50 uL	10 ng/mL
dbcAMP (cAMP)	500 mg/mL	500 uL	250 uL	50 uL	500 ug/mL

Reagent Preparation:

- Doxycycline: 25 mg/mL dissolved in water (10000x stock). Sterilize with a 0.22 µm filter. Store at -20°C. Protect from light.
- Puromycin: 1.25 mg/mL dissolved in water (1000x stock). Sterilize with a 0.22 um filter. Store at -20C.
- Hygromycin: 50 mg/mL (250x stock) Stored at 4C
- CNTF: Reconstitute at 10 µg/ml in sterile 10 mM sodium phosphate containing 0.1% BSA. Aliquot and store at -20°C.
- bFGF: Reconstitute at 1 mg/ml in sterile 5mM Tris, pH 7.6 containing 0.1% BSA. Aliquot and store at -20°C.
- BMP4: Reconstitute at 10 µg/ml in sterile 4 mM HCl containing 0.1% BSA. Aliquot and store at -20°C.
- dbcAMP: Reconstitute at 100 mg/ml in sterile water. Aliquot and store at -20°C. Protect from light.
- N-acetyl-cysteine: Reconstitute at 50 mg/ml in sterile water. Aliquot and store at -20°C.
- Heparin-binding EGF-like growth factor: Reconstitute at 50 µg/ml in sterile PBS containing 0.1% BSA. Aliquot and store at -20°C.

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Day -2: Cell Plating

1. Dissociate hiPSC with with Accutase
2. Replate 3e5 cells per well of a Matrigel-coated 6-well plate (6e5 was way too many)
    a. 3 wells per cell line
3. Plate cells in Stemflex with THX

Day -1 Evening: Lentivirus Transduction

1. Aspirate medium and add Stemflex, Polybrene, and Viruses:

Reagent	**For 1 well**	**For 27 wells**	
StemFlex	2 mL	13.5 mL (→ 14 mL)	1.5 mL per well already
Polybrene (8 ug/mL final)	1.6 uL	44.8 uL (for 14 mL)	
rtTA virus	12.5 uL	Full tube	Michael Fernando's
TetO.Sox9.Puro virus	2.5 uL	Full tube	Pablo's Viruses
TetO.NfiB.Hygro virus	3.3 uL	Full tube	

Day 0 Morning:

- Aspirate virus medium and add 2 mL/well of fresh StemFlex with:
    - 1:10,000 Docycycline (Dox) ([C_f] = 2.5 ug/mL)

For 27 wells:
28 mL + 2.8 mL Dox (times 2)

Day 1:

- Aspirate medium and add 2 mL/well of Expansion Medium with:
    - 1:10,000 Dox ([C_f] = 2.5 ug/mL)
    - 1:1,000 Puro ([C_f] = 1.25 ug/mL)
    - 1:250 Hygro ([C_f] = 200 ug/mL)

For 27 wells: two conicals of:
28 mL Expansion Medium
+ 2.8 uL Dox
+ 28 uL Puro
+ 112 uL Hygro

Day 2:

- Aspirate medium and add 2 mL/well of Expansion Medium with:
    - 1:10,000 Dox ([C_f] = 2.5 ug/mL)
    - 1:1,000 Puro ([C_f] = 1.25 ug/mL)
    - 1:250 Hygro ([C_f] = 200 ug/mL)

For 27 wells: two conicals of:
28 mL Expansion Medium
+ 2.8 uL Dox
+ 28 uL Puro
+ 112 uL Hygro

Day 3a:

- Expand cells 1/6 in Expansion Medium with THX and Dox
    - Can probably get away with 1/3 split but most cell lines do expand to fill the 1/6 split
- 3 wells → 3 6-well plates
- Need 325 mL Expansion Medium + 325 uL THX + 32.5 uL Dox

Day 3b:

- Aspirate medium and add 2 mL/well of ¾ Expansion Medium and ¼ FGF Medium with:
    - 1:10,000 Docycycline (Dox) ([C_f] = 2.5 ug/mL)

Day 4:

- Aspirate medium and add 2 mL/well of ½ Expansion Medium and ½ FGF Medium with:
    - 1:10,000 Docycycline (Dox) ([C_f] = 2.5 ug/mL)

Day 5:

- Aspirate medium and add 2 mL /well of ¼ Expansion Medium and ¾ FGF Medium with:
    - 1:10,000 Docycycline (Dox) ([C_f] = 2.5 ug/mL)

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Day 6:

- Aspirate medium and add 2 mL/well of FGF Medium with:
  - 1:10,000 Docycycline (Dox) ($[C_f]$ = 2.5 ug/mL)
- Note from DRJ: Can replate cells here instead of day 7, just change full FGF media for day 7
- Note from Pablo: Can treat cells today with 4 uM Ara-C to kill off proliferative cells that aren't iAstros

Day 7: Replate or Freeze

1. Dissociate cells with Accutase and pellet at 300g for 5 minutes
2. Replate cells in Matrigel-coated coverslips, petri dishes, or wells as required for experiments
   a. OG Recommendations: 20K in a 96-well, 150K for 24-well, 500K in a 12-well
   b. If counting with Cellometer, you should plate as follows:
      i. 4e4 cells per 12 well
      ii. 2e4 cells per 24 well
      iii. 1e4 cells per 48 well
      iv. 5e3 cells per 96 well
   - FGF Medium with 1:10,000 Docycycline (Dox) ($[C_f]$ = 2.5 ug/mL) and 1:1000 THX
3. Alternatively, freeze down cells in FGF media + 10% DMSO in a Mr. Frosty
   a. When thawing out: thaw out in FGF media with 1:1000 THX and change FGF media next day Plate for experiments the day after (try this out and see how it goes)

Day 8:

- Aspirate medium and add ¾ FGF Medium and ¼ Maturation Medium with:
  - 1:10,000 Docycycline (Dox) ($[C_f]$ = 2.5 ug/mL)
- Note from DRJ: Can hold off on switching the cells to MM by at most 3 days

Day 9:

- Aspirate medium and add ½ FGF Medium and ½ Maturation Medium with:
  - 1:10,000 Docycycline (Dox) ($[C_f]$ = 2.5 ug/mL)

Day 10:

- Aspirate medium and add ¼ FGF Medium and ¾ Maturation Medium with:
  - 1:10,000 Docycycline (Dox) ($[C_f]$ = 2.5 ug/mL)

Day 11:

- Aspirate medium and add Maturation Medium
  - No more Dox required

Day 13-21:

- Change half of the medium every 2-3 days
  - Account for evaporation
    - 24 well plate: Remove 200 uL, add 250 uL
    - 48 well plate: Remove 100 uL, add 125 uL

Day 21:

- Astrocytes are ready for experiments