Using NGR (induced neuron) lines: Culture, maintenance and expansion

NeuroHub Seminar

Christina Muratore, Ph.D

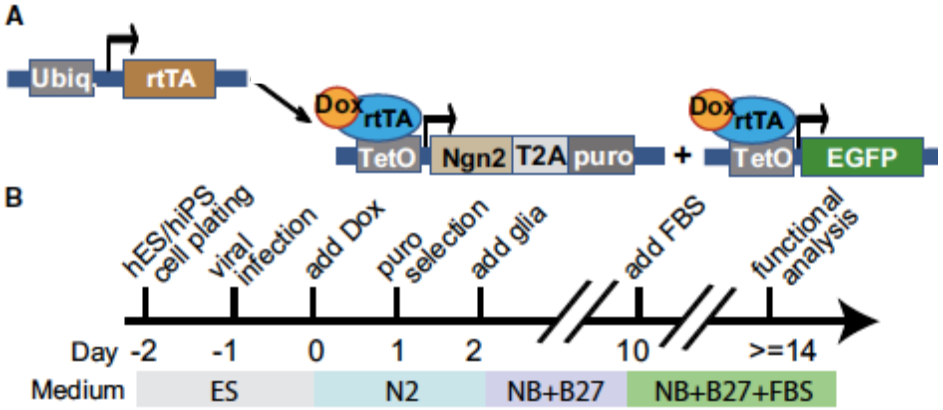
May 22, 2018, 2:30pm-3:30pm

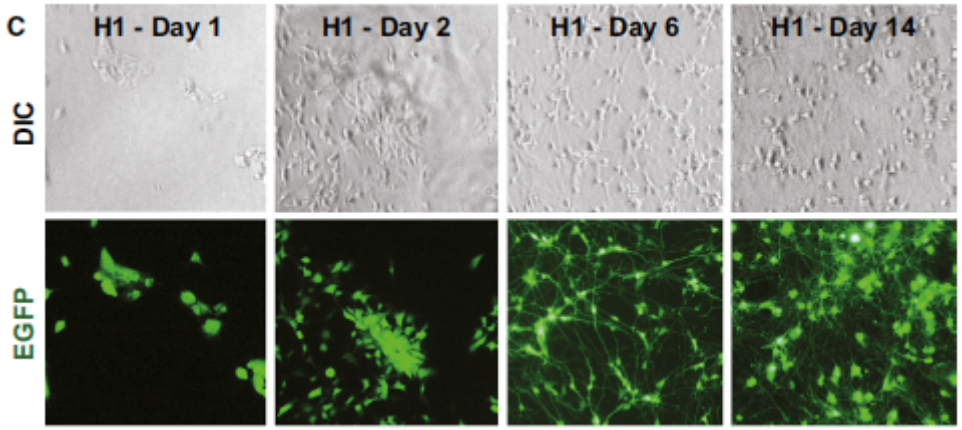
BTM 10004

Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells

Neuron, 2013

Yingsha Zhang,[1] ChangHui Pak,[1,6] Yan Han,[1,6] Henrik Ahlenius,[3,4] Zhenjie Zhang,[5] Soham Chanda,[1,3,4] Samuele Marro,[3,4] Christopher Patzke,[1] Claudio Acuna,[1] Jason Covy,[1] Wei Xu,[1,2] Nan Yang,[3,4] Tamas Danko,[1,3] Lu Chen,[5] Marius Wernig,[3,4] and Thomas C. Südhof[1,2,5,*]





Further optimized by Eggan and Young-Pearse labs

Quick Terminology

- iPSCs – pluripotent, un-altered, cannot make iNs
- NGR (NCR) iPSCs – have been transduced with virus to make iNs, still pluripotent
  - Neurogenin 2 - TetO-Ngn2-Puro
  - GFP - TetO-GFP
  - Reverse tetracycline transactivator - Ubq-rtTA
- D4 iNs – with Dox, no longer pluripotent, on track to be neurons, derived from NGR or NCR lines

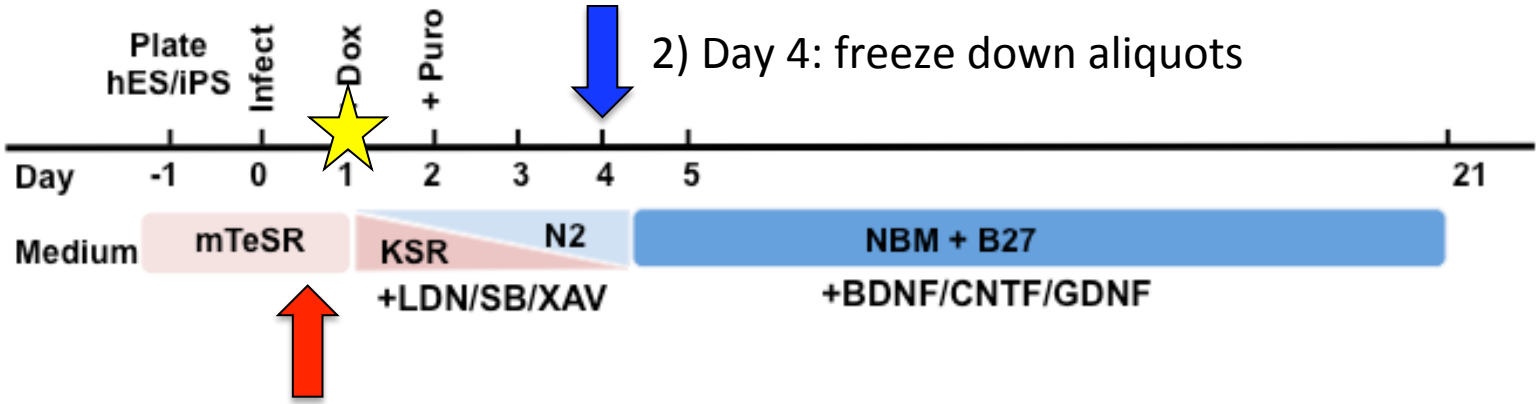
Parkinson's Disease iPSC Lines

iPSC NeuroHub
An open source ARCND repository

iPSC Line	Genotype	Sex and Age at Tissue Collection	Status and Available Materials	Additional Associated Lines and Details	Original Source	Approvals Necessary
EDi001-A	SNCA triplication	Female (55)	• Available ◦ iPSCs ◦ NGR iPSCs	• isogenic control (EDi001-A-4)	EBiSC	None (with MTA in place)
ND50085	SNCA A53T	Female (51)	In house, needs to be expanded	• mutation line went through editing process, but no correction made • isogenic control (ND50086)	NINDS	Some labs may need to be added to MTA

Induced Neurons (iNs)

- Forced expression of Ngn2 (lentivirus)
- Rapid induction (14-28 days)
- Specifically layer 2/3 excitatory neurons, no astrocytes
- Easily scalable



Optimized by Eggan and Young-Pearse labs

Protocol adapted from Zhang et al. Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells. Neuron. 2013;78(5):785-798.

Making and Expanding NGR iPSCs

- Expand NGR iPSCs before differentiating
- 2M cells/10 cm plate
- Split with accutase (single-cells)

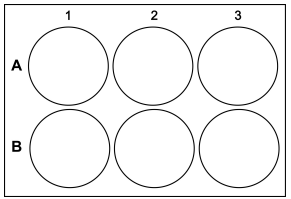
Virus available for purchase!

$318.75 for set of 3 → 10-12 lines from 1 aliquot

iPS Cells maintenance

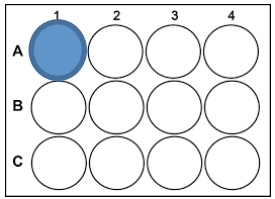
NGR or NCR lines

Maintenance of iPSC cells
mTeSR media



Matrigel-Growth Factor Reduced (GFR)
Corning #354230

(mTeSR media)



Matrigel-GFR
Corning #354230

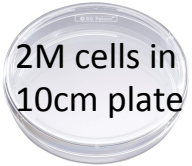
NGR or NCR lines
(passage 1)

mTeSR media

Matrigel-GFR
Corning #354230

5-7 days

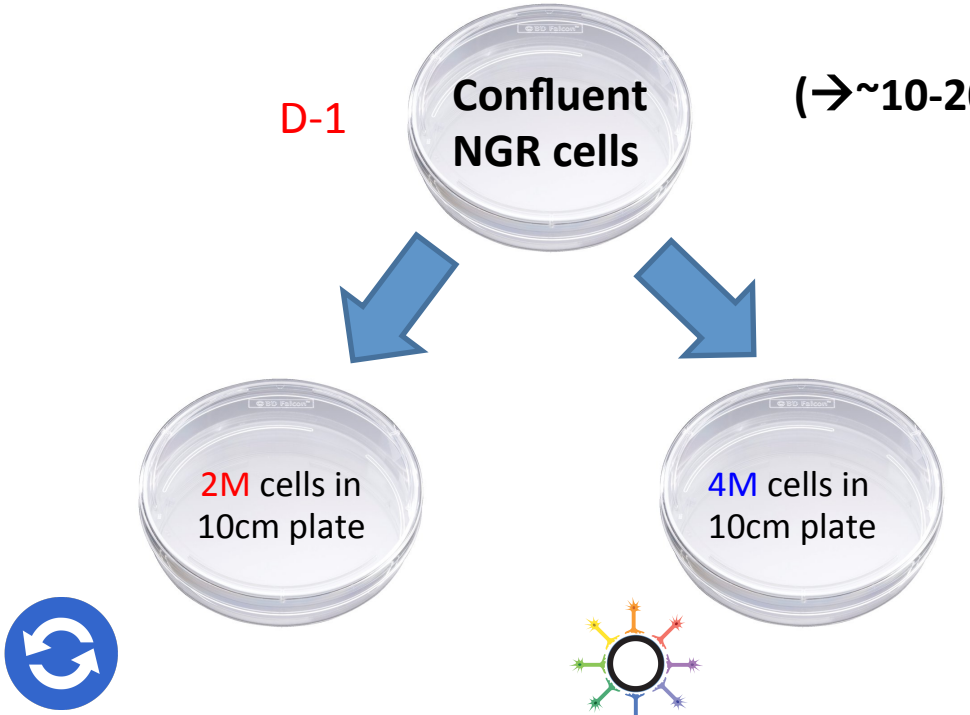
Expansion of NGR or NCR lines
mTeSR media



Matrigel-GFR
Corning #354230

"D-1"

Workflow


D-1
Confluent NGR cells
(→~10-20M cells/10cm plate)
2M cells in 10cm plate
4M cells in 10cm plate
For "D-1" maintenance
For "D0" plating and further differentiation

Methods and Reagents

NGR iPSC culture on Matrigel-GFR with mTeSR media – for splitting and plating

- ***mTeSR media + 1:1000 Rock inhibitor (Fc=10uM)
- Accutase:PBS (1:3) + 1:1000 Rock inhibitor (Fc=10uM)
- warm Matrigel (GFR) pre-coated plates

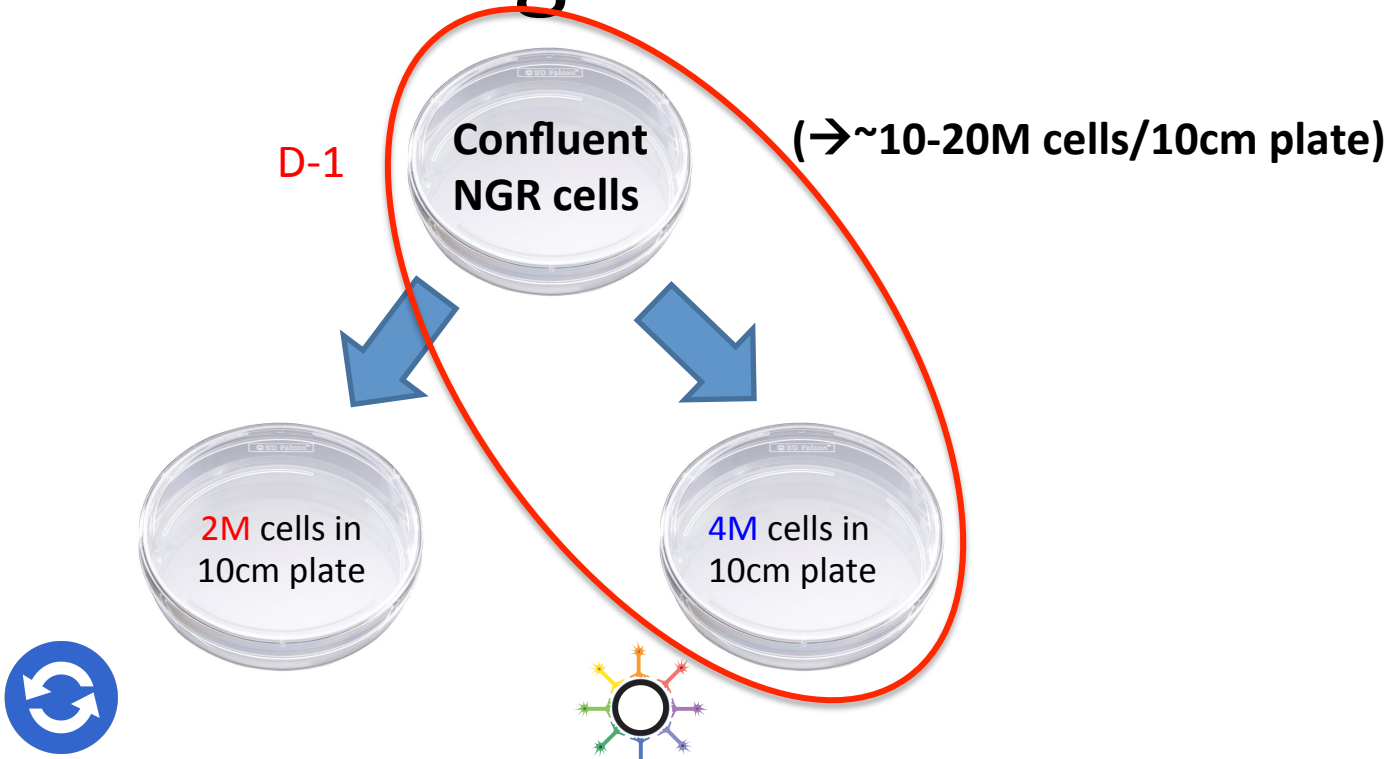
- ❖ warm up mTeSR media in water bath or heating beads
- ❖ bring Accutase from 4°C to hood up to room temp before using. (* Do Not warm up Accutase in 37°C; this will inactivate it.)
- ❖ warm up Matrigel (GFR) pre-coated plates

***Alternatively, can use StemFlex media

Splitting cells (when confluent, after ~5-6 days)

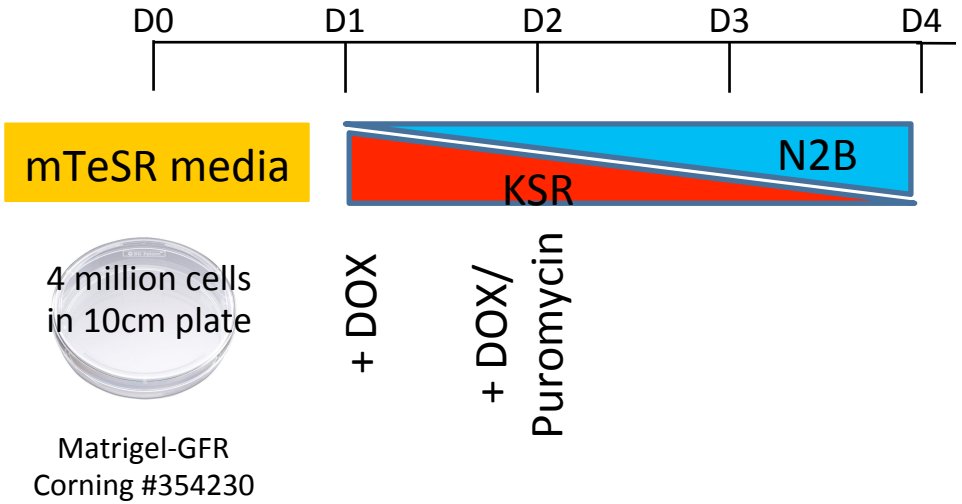
1. Wash cells with PBS, and then treat cells with 3mL/10cm plate 3x diluted accutase (in PBS) for 5-10 min or longer at 37^{o}C incubator.
   a. Incubation time depends on cell line!
2. Under the microscope, **tap** the culture plate to help detach cells from plate (cells are ready when they **round up**).
3. Add 2-3mL of fresh mTeSR media with 10 µM ROCK inhibitor to collect cells.
   a. Pipette in conical tube.
   b. Aliquot some for counting (10-15uL).
4. Centrifuge cells at 500xg, 5 min.
5. Resuspend cells into desired density for plating or freezing.
   a. 4M cells/10cm plate for iN differentiation (D0)
   b. 2M cells/10 cm plate for maintenance/expansion (D-1)

Banking D4 Cells



Banking D4 Cells

Differentiation NGRs/NCRs to inducible neurons (iN)



Day 4: freeze down aliquots
~20-30M cells/10cm plate

iN Differentiation

Day 0: plating **4x10^6**/10cm GFR-Matrigel coated plate in mTeSR media/ROCK inhibitor

Day 1: 7ml of KSR media + DOX (2µg/ml) (1:10k) to induce NGN2 and GFP expression

Day 2: 7ml of KSR:N2B (1:1) + DOX (2µg/ml) (1:10k) + puromycin (puromycin concentration depends on cell line - 2000x dilution (5 µg/ml) is good for most lines).
* Cells without NGN2/puro resistance will die off.

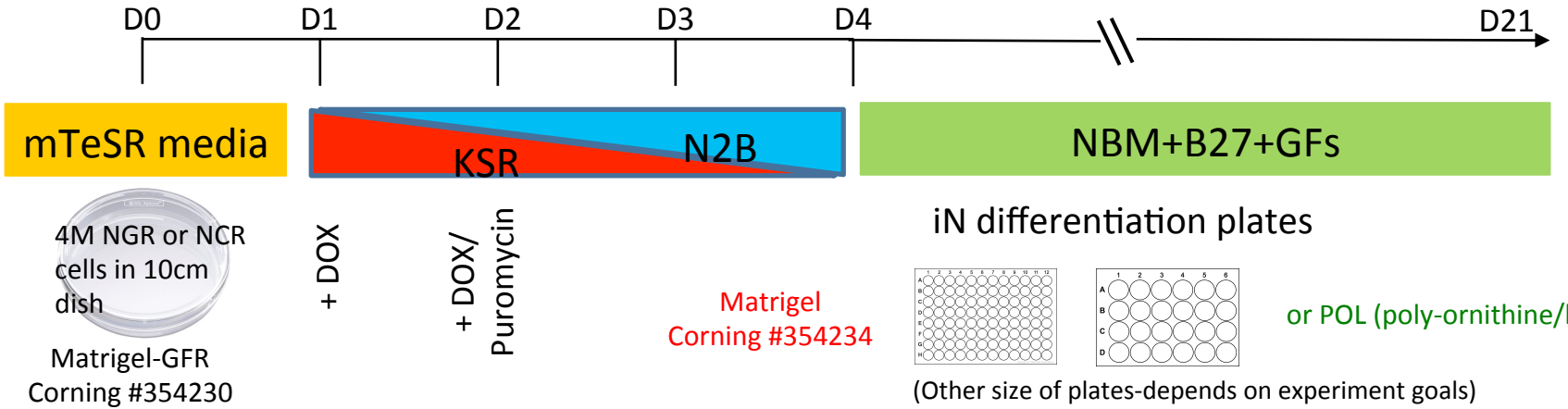
Day 3: 7ml of N2B media + B27 (1:100)+ DOX (2µg/ml) (1:10k) + puromycin

Day 4: **NBM media + B27 (1:50)+ DOX (2µg/ml) (1:10k) + puromycin + BDNF/CNTF/GDNF (1:1000) + ROCK inhibitor**

- Wash iN-day4 cells with PBS and then dissociate cells with 3mL of 3x diluted Accutase for 5-10 min. (Incubation time varies with cells lines)
- Add 3-5mL of iN-d4 media (NBM) to triturate cells; aliquot some for cell counting.
- Resuspend in NBM for desired cell density.

* Freeze down 1x10^6/cryo vial as iN-d4 stock (Freezing media: iN media (NBM/growth factors): FBS: DMSO= 5:4:1)
* Or plate onto Matrigel-coated differentiation plate.

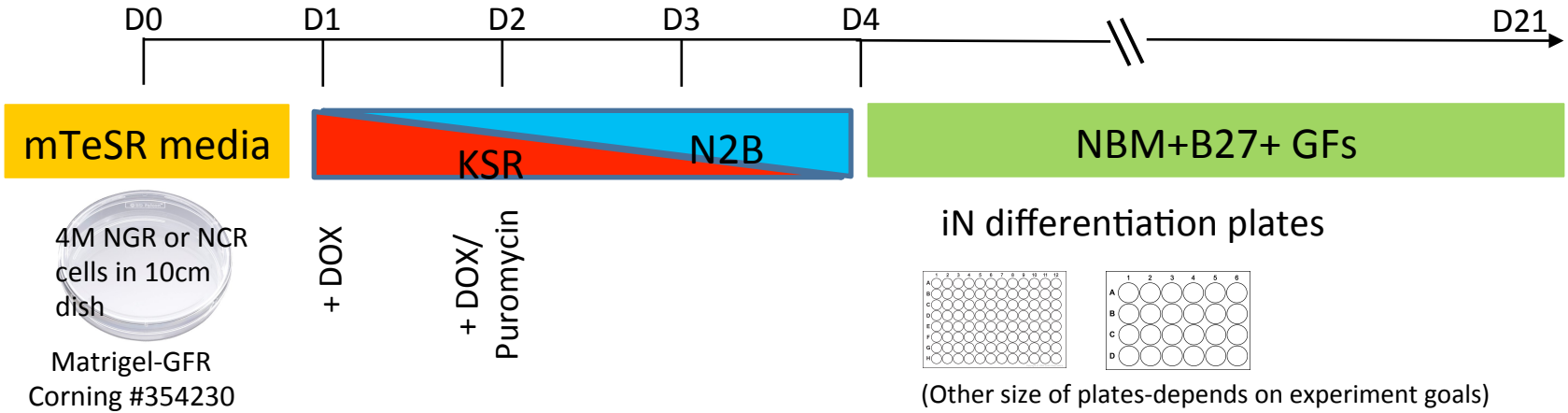
Thawing D4 iN aliquots for differentiation to inducible neurons (iNs)



Day 4: **NBM media + B27 (1:50)+ DOX (2µg/ml) (1:10k) + puromycin + BDNF/CNTF/GDNF (1:1000) + ROCK inhibitor (1:1000)**

- Warm up Matrigel (#354234) coated differentiation plates, NBM with growth factors.
- **Thaw out frozen iN d4 cells** from liquid nitrogen fast and add fresh and warm media into cryovial.
- Take aliquot for cell counting (viability up to 90%).
- Spin down cell suspension (250xg, 5 min).
- Resuspend in NBM with GFs for desired cell density.
- Plate 40,000-50,000 cells/cm2 (for example: 16K for 96wells) (differentiation plate size and density is experimental goal -dependent).

Thawing D4 iN aliquots for differentiation to inducible neurons (iNs)



Day 5 and beyond: NBM + B27 (1:50)+ DOX (2µg/ml) (1:10k) + puromycin + BDNF/CNTF/GDNF (1:1000) – fresh prepared

Day 5: add half of fresh media gently

Day7: 2/3 media change (for example, taking out 70 µl from 96wells-plate and add fresh 100 µl of fresh media.)

***Feeding once a week is acceptable with lower densities.

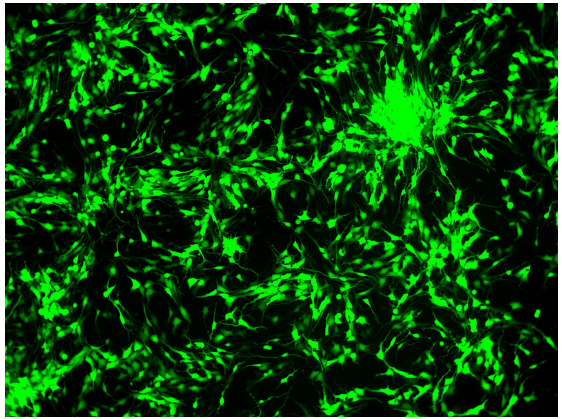
If cells are cultured more than d21-28, astrocytes co-culture is necessary.

Astrocyte Co-culture

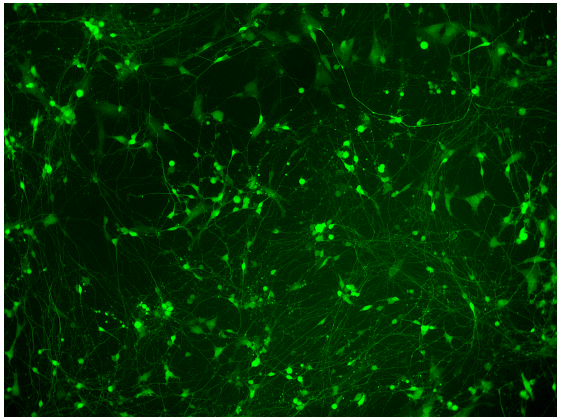
- Can help stabilize iNs for extended culture
- Aids in maturation
- Astrocyte options
  - Primary astrocytes
  - iPSC-derived astrocytes
- Supplement NBM with 5% FBS
- No puromycin
- Plate D4 neurons first, treat with with puro for 2-3 days, then add astrocytes

NGR Differentiation Image Timeline

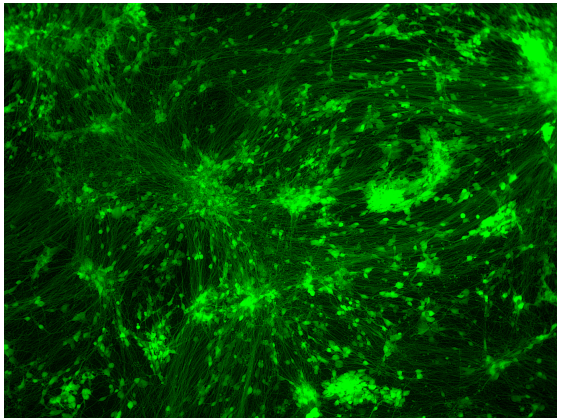
D5



D10



D16



D28