| **Midbrain dopamine neurons from hESCs** | Prepared by:<br>Mark Tomishima<br>tomishim@mskcc.org |
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| Adapted from | Kriks et al., 2012 |
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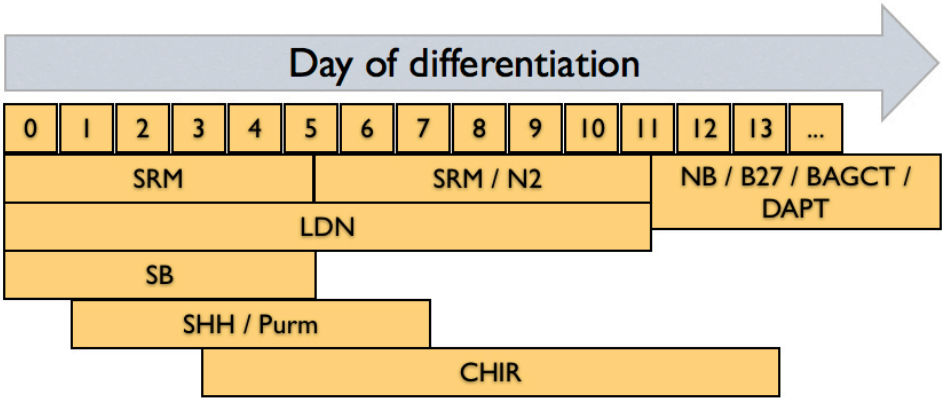
***Introduction*:**

The Kriks et al. protocol has finally achieved what we believe are genuine midbrain, dopamine neurons. Perrier et al. (2004) described a method to make dopamine neurons that we initially thought were mDA neurons. Later work by developmental biologists, however, discovered additional markers of these neurons that the Perrier cells lacked (e.g., FOXA2/TH). The Perrier protocol used neural rosettes as a patterning intermediate to make dopamine neurons. Later work in vivo demonstrated that rodent mDA neurons are derived from the floor plate, a cell type at the base of the neural tube that is not normally neurogenic – its usual role is to produce SHH that helps pattern the neural tube. Fasano et al. (2010) demonstrated a protocol to make floor plate from hESCs but the protocol was not efficient at making mDA neurons. Floor plate is achieved by very early, pre-rosette exposure to high levels of SHH. The Kriks et al. protocol modified this protocol to include early WNT activation, and this turned out to be the key for driving the progenitors into mature mDA neurons. Importantly, these neurons can be transplanted into the brain and retain the mDA neuron phenotype: something that the Perrier et al. cells could not do.

***Flowchart*:**



**Instructions:**

1. Accutase treat hESCs (on an MEF monolayer) for 30-45 minutes, until all colonies are single cells.
2. Pipet Accutase into 15 ml conical with hESC media.
   *at least two volumes of hESC to one volume of Accutase*
3. Centrifuge for 5 minutes at 200 xg, room temperature.
4. Gelatin treat a new tissue culture dish during the centrifugation.
5. Resuspend cells in hESC media with 10 µM Y-27632.
6. Aspirate gelatin from culture dish.
7. Add hESCs (from step 5) to gelatinized dish for 1 hour at 37˚C in the incubator.
   *MEFs preferentially attach to the gelatinized dish, giving a nearly pure population of hESCs after the incubation*
8. While incubating, prepare a Matrigel-coated plate (1:20 in DMEM or hESC media).
9. After the hour, collect the non-adherent cells from the incubator and gently wash the dish. Centrifuge cells as above.
10. Count cells and plate on Matrigel-treated dishes in hESC media conditioned on high-density MEFs with 10 ng/ml FGF2 and 10 µM Y-27632. Plate at 200,000 cells/cm2. At this density, cells should be confluent overnight. If they are not confluent, continue expansion in CM until they are and then induce differentiation.

**Begin differentiation**

11. Day 0 - initiation. Aspirate hESC media and add SRM with 100 nM LDN193189 / 10 µM SB431542
12. Day 1 - SRM / LDN / SB with 100 ng/ml SHH (C25II - see materials) and 2 µM Purmorphamine
13. Day 2 - SRM / LDN / SB / SHH / Purm
14. Day 3 - SRM / LDN / SB / SHH / Purm / 3 µM CHIR 99021
15. Day 4 - no feed
16. Day 5 - 75% SRM / 25% N2 with LDN / SHH / Purm / CHIR
17. Day 6 - no feed
18. Day 7 - 50% SRM / 50% N2 with LDN / CHIR
19. Day 8 - no feed
20. Day 9 - 25% SRM / 75% N2 with LDN / CHIR
21. Day 10 - no feed
22. Day 11 - NeuroBasal/B27 with CHIR / BDNF / AA / GDNF / cAMP / TGFB3 / 10 µM DAPT
    *(put poly-L-ornithine solution on plate overnight in incubator)*

23. Day 12 - no feed
    *(aspirate poly-ornithine, wash 3 times with PBS, and add fibronectin/laminin overnight in incubator)*
24. Day 13 - Passage 1:1 onto poly-L-ornithine/fibronectin/laminin-coated dishes with 30-45 minutes of Accutase treatment. Spin down in NB/B27, and resuspend in NB/B27 with BAGCT and DAPT (same as above without CHIR)
25. Day 14 - no feed
26. Day 15 - from here, keep the same media composition and feed every other day.
27. Between D20-25 when cells become bipolar and make space on the dish, passage them again to poly-L-ornithine/fibronectin/laminin-coated dishes using Accutase. Replate 300-400K per 24 well/well or 2-3 million per 6 well/well with "center plating" (see FAQ)

**Materials:**
SB431542, Tocris 1614
LDN193189, Stemgent 04-0074
Purmorphamine, Calbiochem 540220
CHIR99021, Stemgent 04-0004
SHH (C25II), R&D Systems 464-SH
BDNF, R&D Systems 248-8D
Ascorbic acid, Sigma A-4034
GDNF, Peprotech 450-10
dbcAMP, Calbiochem 28745
TGF-B3, R&D Systems 243-B3
DAPT, Tocris 2634
Y-27632 dihydrochloride (Tocris Bioscience, cat. no. 1254) dissolved in filtered water to 10 mM (1000x stock).

Accutase, Innovative Cell Technologies AT104

poly-L-ornithine (15 ug/ml) overnight, then
laminin (1 ug/ml)
fibronectin (2 ug/ml) (overnight)
All diluted in DPBS before adding to the plate.

Matrigel

C refrigerator. Prepare 1 mL aliquots in a 50 mL centrifuge
t - C. Matrigel must be thawed slowly to

prevent gelatinization. Chilled pipettes and 50 mL centrifuge tubes should be used when making aliquots of the Matrigel.

Mouse embryonic fibroblasts (MEFs, GlobalStem, Inc., GSC-6001M)

**N2 media** contains DMEM/ F12 powder (Gibco/Invitrogen, cat no. 12500-062) in 550 mL of distilled water. 1.55 g of glucose (Sigma, cat. no. G7021), 2.00 g of sodium bicarbonate (Sigma, cat. no. S5761), putrescine (1 ml aliquot of 1.61 g dissolved in 100 mL of distilled water; Sigma, cat. no. P5780), progesterone (20 uL aliquot of 0.032g dissolved in 100 mL 100% ethanol; Sigma, cat. no. P8783), sodium selenite (60 uL aliquot of 0.5 mM solution in distilled water; Bioshop Canada, cat. no. SEL888), and 100 mg of transferrin (Celliance/Millipore, cat. no. 4452-01) are added. 25 mg of powdered insulin (Sigma, cat. no. I6634) is added to 10 mL of 5 mM NaOH and is shaken until completely dissolved. The solubilized insulin is added to the media, and double-distilled water (with a resistance of 18.2 MΩ) is added to a final volume of 1000 mL before sterile filtration.

****Recently, the Studer and Tomishima labs have had problems with the supply chain for this media. This has caused us to explore new sources, and many are now using Neurobasal with N2 supplements and B27 (without retinoic acid) and L-glutamine.*

**hPSC media**
780 ml DMEM:F12 (Invitrogen, cat.# 11330-032)
200 ml Knockout serum replacement (KSR; Invitrogen, cat. no. 10828-028)
5 ml L-glutamine (200 mM, Invitrogen, cat.# 25030-081)
10 mL MEM non-essential amino acids (MEM NEAA; Invitrogen, cat.# 11140-050)
1 mL of 2-mercaptoethanol (Invitrogen, cat.# 21985-023)
The medium is sterile filtered in the hood and FGF2 is added after filtration to a final concentration of 6 ng/ml.

Recombinant FGF2 (R&D Systems, cat.# 233-FB-001MG/CF) dissolved in PBS with 0.1% BSA to 100 μg/ml.

**SRM media** (a.k.a. KSR media) contains 820 mL of Knockout DMEM (Invitrogen; cat. no. 10829-018), 150 mL Knockout Serum Replacement (Invitrogen, cat. no. 10828-028), 10 mL L-glutamine (200 mM, Invitrogen, cat. no. 25030-081), 10 mL MEM NEAA (Invitrogen, cat. no. 11140-050), and 1 mL of 2-mercaptoethanol (Invitrogen, cat. no. 21985-023).

**MEF conditioned media (CM)** is harvested from MEF coated flasks. MEFs are plated at a density of 50,000 cells/cm2 in a T225 flask in MEF media. The next day, the cells are washed once with PBS before adding 100 mL of hESC media. Incubate media with MEFs for 24 hours before removal. T

C for less than two weeks. Additional hESC media can be conditioned daily for up to ten days on the same flask of feeders. Just before using, FGF2 is added to CM to a final concentration of 10 ng/mL, hereafter called complete CM (cCM).

**FAQs:**

**Q:** How do I perform the "center plating" procedure?
**A:** This is done to keep the local cell density high and to keep the monolayer from peeling off at the edge of the dish. To perform this procedure, aspire the fibronectin/laminin solution and "chase the rainbow" - move the aspirator just over the surface of the dish to enhance the drying. After the dish is completely dry, allow it to dry without the lid on in the hood for another 10-20 minutes. The cell suspension is placed in 1/10 of the media that will be used ultimately and is carefully and slowly added to the center of the dish. The media should "bead" on the surface of the dish, confining the cells to a small area in the center of the dish. After 10 minutes without moving the dish in the hood, the remaining 9/10 of media is carefully added around the perimeter of the dish, taking care not to disturb the drop. Carefully move the dish to the incubator

**Q:** I see cell death during days 7-11. What am I doing wrong?
**A:** Make sure your cells are mycoplasma negative. And, we have seen this with certain N2 batches. Recently, we've had more success using NeuroBasal/B27 (no RA) and N2 supplements. You might want to try this medium if you experience survival problems.

**Q:** I see cell death from days 16-20. What am I doing wrong?
**A:** You might have to feed every day late in the differentiation to keep the culture robust.