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Efficient Differentiation of Postganglionic Sympathetic Neurons Using Human Pluripotent Stem Cells

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Nadja Zeltner, PhD
Assistant Professor

Athens, November 25th, 2019

Phillip Steindel, Ph.D., Review Editor

Dear Dr. Steindel,

Please find enclosed our revised manuscript entitled **" Efficient Differentiation of Postganglionic Sympathetic Neurons Using Human Pluripotent Stem Cells under Feeder-free and Chemically Defined Culture Conditions"** by Hsueh Fu Wu and Nadja Zeltner. We have now revised the manuscript, we adressed the editorial and reviewers comments to the best of our capabilities. We apologize for the delayed submission.

Our manuscript is not under submission in any other journal and all authors agreed to the final version of the manuscript. There are no conflicts of interest for any authors.

Thank you for your consideration of the manuscript and please do not hesitate to contact me with additional questions.

Sincerely,

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TITLE:

Efficient Differentiation of Postganglionic Sympathetic Neurons Using Human Pluripotent Stem Cells under Feeder-free and Chemically Defined Culture Conditions

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KEYWORDS:

human pluripotent stem cells, neural crest, sympathetic neurons, autonomic nervous system, sympathetic nervous system, feeder-free, chemically defined stem cell culture

SUMMARY:

In this protocol, we describe a stable, highly efficient differentiation strategy for the generation of postganglionic sympathetic neurons from human pluripotent stem cells. This model will make neurons available for the use of studies of multiple autonomic disorders.

ABSTRACT:

Human pluripotent stem cells (hPSCs) have become a powerful tool for disease modeling and the study of human embryonic development in vitro. We previously presented a differentiation protocol for the derivation of autonomic neurons with sympathetic character that has been applied to patients with autonomic neuropathy. However, the protocol was built on Knock Out Serum Replacement (KSR) and feeder-based culture conditions, and to ensure high differentiation efficiency, cell sorting was necessary. These factors cause high variability, high cost, and low reproducibility. Moreover, mature sympathetic properties, including electrical activity, have not been verified. Here, we present an optimized protocol where PSC culture and differentiation are performed in feeder-free and chemically defined culture conditions. Genetic markers identifying trunk neural crest are identified. Further differentiation into postganglionic sympathetic neurons is achieved after 20 days without the need for cell sorting. Electrophysiological recording further shows the functional neuron identity. Firing detected from our differentiated neurons can be enhanced by nicotine and suppressed by the adrenergic receptor antagonist propranolol. Intermediate sympathetic neural progenitors in this protocol can be maintained as neural spheroids for up to 2 weeks, which allows expansion of the cultures.

In sum, our updated sympathetic neuron differentiation protocol shows high differentiation efficiency, better reproducibility, more flexibility, and better neural maturation compared to the previous version. This protocol will provide researchers with the cells necessary to study human disorders that affect the autonomic nervous system.

INTRODUCTION:

Postganglionic sympathetic neurons (symNs) belong to the autonomic nervous system (ANS) and have multiple important roles in responding and regulating homeostasis of the body independent of consciousness. For example, stress stimulates symNs and evokes the fight-or-flight response that leads to an increase in heart rate, blood pressure, and sweating. SymNs are affected in multiple human disorders due to genetics, toxicity/injury, or as companions to other diseases. An example of a genetic neuropathy is the childhood disorder Familial Dysautonomia (FD), where a severe dysregulation of symNs causes dysautonomic crisis, evident by sweating, blotching of the skin, vomiting attacks, hypertension, and anxiety[1]. An example of toxicity is chemotherapy treatment, which has been reported to have toxic side effects on autonomic neurons[2]. It is known that autonomic denervation and hyper-innervation can both lead to, or accompany, diseases such as Parkinson's disease or hypertensive renal disease[3,4]. Thus, being able to conduct research and understand the mechanisms of symN biology and defects in the context of disease is beneficial for the search of novel and effective treatments.

Anatomy

The peripheral nervous system branches into sensory and autonomic divisions. The afferent nerves of the sensory nervous system are responsible for sensation of pain and touch, whereas the ANS is responsible for relaying information from all organs to the brain. The ANS is divided into the enteric nervous system, innervating the gastrointestinal tract, the parasympathetic nervous system, which is important for relaxation, and the sympathetic nervous system (SNS), which is important for activation/regulation of organs. The SNS adapts a two-neuron system[5]. Preganglionic sympathetic neural axons in the spinal cord first project to the sympathetic ganglia, where postganglionic symN cell bodies are located. These neurons then send long projections to innervate the target tissues of every organ in the body. Signals transmitted by preganglionic neurons are cholinergic, whereas postganglionic symNs are adrenergic and thus express norepinephrine (NE) as their main neurotransmitter. There are few notable exceptions of postganglionic, sympathetic neurons that are cholinergic, including the ones innervating blood vessels. Adrenergic postganglionic neurons express the enzymes tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AAAD), dopamine β-hydroxylase (DBH), and monoamine oxidase (MAO-A), all responsible for generating and metabolizing NE. Furthermore, they express the NE recycling transporters and/or receptors α-adrenergic receptor (ADRA2), β-adrenergic receptor (ADR2B), norepinephrine transporter (NET1), and vesicular monoamine transporter (VMAT1/2).

Development

During embryonic development symNs are derived from the neural crest (NC), which emerges between the neural tube and overlaying ectoderm[6], and can differentiate into multiple cell lineages, including melanocytes, osteoblasts, adipocytes, glia, enteric neurons, sensory neurons,

and autonomic neurons[7]. Neural crest cells (NCCs) are highly migratory cells that take several routes through the embryo. At this early stage of NC development, the cells express the markers SNAIL1/2, FOXD3, and SOX10[8-11]. The migration route together with the axial location they adopt determines the NC subtype into which they will develop. These NC subtypes can be distinguished by their specific HOX gene expression: Cranial NCCs do not express HOX genes, vagal NCCs express HOX 1–5, trunk NCCs express HOX 6–9, and sacral NCCs express HOX 10–11[12]. Among them, trunk NCCs are recognized as the main source of symNs. SymN precursors express the transcription factor MASH1/ASCL1[13], which promotes expression of PHOX2B[14] and INSM1[15]. The GATA family of transcription factors is expressed during late sympathetic development. GATA2 and GATA3 are expressed in the symNs, which in turn activates DBH[16]. The transcription factor HAND2 is also important for the expression and maintenance of DBH and TH[17].

HPSCs (e.g., embryonic and induced pluripotent stem cells) are a powerful tool[18] to recapitulate developmental paradigms and generate symNs that can then be employed for disease modeling of various human disorders. Thus, while generating symNs from hPSCs, it is crucial to follow developmental guidelines and assess expression of appropriate markers along the differentiation process.

Previous symN protocol

Few research groups have previously reported the generation of symNs from hPSCs[19-21]. The direct comparison of these protocols to each other and ours was reviewed recently[22]. In 2016[23], we published a differentiation protocol for the generation of autonomic neurons with symN character (**Figure 1A**). This protocol used KSR-based medium, which was used in both the maintenance of undifferentiated hPSCs and cell differentiation. Furthermore, hPSCs were maintained on mouse embryonic fibroblasts (MEF feeder cells). We employed this protocol and PSCs from patients with FD to model the disorder[23]. In 2019, we described a more detailed version of this older protocol[24]. In summary, the neural fate was induced by dual SMAD inhibition[25] to block TGF-β and BMP signaling in the first 2 days. WNT activation using CHIR99021 promoted neural progenitors to become NC cells. On day 11, cells were sorted by FACS for $CD49D^+$ or $SOX10^+$ populations[26,23], which yielded about 40% NC generation efficiency. Thus, sorting was needed to ensure the efficiency and purity for the next steps of differentiation. The NCCs were maintained and amplified as spheroids with the combined treatment of FGF2 and CHIR. After 4 days, the NC spheroids of maintenance were plated and given BDNF, GDNF, and NGF to finish the symN maturation. Although these symNs expressed strong symN markers such as ASCL1, TH, DBH, and PHOX2A, markers for more mature symNs, including expression of the nicotinic acetylcholine receptor (CHRNA3/CHRNB4) and vesicle transporter (VMAT1/2), were low even after 70 days of differentiation. HOX genes in this protocol were not formally tested, and mature neural properties, including electrophysiological activity of the cells, were not verified.

Here, we present an optimized protocol to generate symNs (**Figure 1B**). HPSCs are maintained in feeder-free conditions, on vitronectin (VTN)-coated dishes, using Essential 8 (E8) media[27]. The formula of the differentiation media has been modified at each stage, thereby increasing the percentage of the NC population[28]. The symN maturation can be done on $CD49D^+/SOX10^+$ sorted or unsorted bulk NCC populations. Both show high levels of symN marker expression by day 30.

Moreover, the symNs generated with this protocol are responsive to electrophysiological recording and to treatments with symN activator and inhibitor compounds.

PROTOCOL:

NOTE: The H9 PHOX2B:GFP reporter line was provided by Oh et al.[19]. Some qPCR primers used in this paper were obtained from OriGene Technologies, while a few sequences are obtained from Frith et al.[20,30].

1. Set-up for dish coating, media preparation, and hPSC maintenance

1.1. Dish coating

1.1.1. **Vitronectin (VTN) coating**

1.1.1.1. Place vials of VTN in a 37 °C water bath until fully thawed, then mix thoroughly.

1.1.1.2. For a 100 mm Petri dish, mix 7 mL of 1x phosphate buffered saline (PBS) with 0.5 mg/mL VTN, add VTN solution to the dish, and incubate at room temperature (RT) for 1 h.

1.1.2. **Basement membrane matrix coating**

1.1.2.1. Thaw vials of basement membrane matrix (see **Table of Materials**) on ice at 4 °C overnight.

1.1.2.2. For one well of a 6 well plate, mix 2 mL of DMEM/F12 with 20 µL of 100x basement membrane matrix, add basement membrane matrix solution to the dish, wrap the dish with paraffin film, and store in a clean container at 4 °C overnight. Work as quickly as possible. Coated dishes can be stored in 4 °C for up to 2 weeks.

1.1.3. **Polyornithine (PO)/laminin (LM)/fibronectin (FN) coating**

1.1.3.1. On the first day, for one well of a 24 well plate, mix 15 µg/mL of PO with 1 mL of 1x PBS, incubate at 37 °C, 5% CO_2 overnight. Thaw both LM and FN at -20 °C overnight and store at 4 °C until fully thawed.

1.1.3.2. On the second day, aspirate PO solution, wash the wells 2x with 1x PBS, add 1 mL of 1x PBS containing 2 µg/mL of LM and 2 µg/mL of FN and incubate at 37 °C in 5% CO_2 overnight. At this point the dish with the LM/FN solution can be kept in the incubator for months as long as it does not dry out. Add more 1x PBS to prevent the dish from drying out.

1.2. Media preparation

1.2.1. **Prepare the Essential 8 medium (E8)** by thawing one bottle of E8 supplement at 4 °C overnight. Mix the supplement with 500 mL of E8 medium and antibiotics if needed.

NOTE: Working E8 solution should be used up within 2 weeks.

1.2.2. **Prepare the hPSC freezing medium by** mixing 90 mL of complete E8 medium with 10 mL of DMSO for a total volume of 100 mL. Filter sterilize.

1.2.3. **Prepare the day 0 to day 1 differentiation medium** by mixing 100 mL of essential 6 (E6) medium with 10 µM SB431542, 1 ng/mL BMP4, 300 nM CHIR99021, and 10 µM Y27632 for a total volume of 100 mL.

1.2.4. **Prepare the day 2 to day 10 differentiation medium** by mixing 100 mL of E6 medium with 10 µM SB and 0.75 µM CHIR99021 for a total volume of 100 mL.

1.2.5. **Prepare the day 10 to day 14 spheroid medium by** mixing neurobasal medium with 2 mL of B27 (50x), 1 mL of N2 (100x), 2 mM L-Glutamate, 3 µM CHIR99021, and 10 ng/mL FGF2 for a total volume of 100 mL.

1.2.6. **Prepare the day 14 to day 28 medium for spheroid long term maintenance** by adding 0.5 µM of fresh RA to the day 10 to day 14 spheroid medium for every feeding.

NOTE: Always keep RA at -80 °C.

1.2.7. **Prepare the SymN maturation medium** by mixing neurobasal medium with 2 mL of B27 (50x), 1 mL of N2 (100x), 2 mM L-glutamate, 25 ng/mL GDNF, 25 ng/mL BDNF, 25 ng/mL NGF, 200 µM ascorbic acid, and 0.2 mM dbcAMP for a total volume of 100 mL. The solution should be used within 2 weeks. Before each feeding, add 0.125 µM fresh RA. This solution is used from day 14 (option 1) or day 28 (option 2).

1.2.8. **Prepare the FACS buffer** by mixing DMEM with 2% FBS, 2 mM L-glutamate and antibiotics if needed for a total volume of 100 mL.

1.3. hPSC maintenance

1.3.1. Thawing and keeping hPSCs

1.3.1.1. Prepare one VTN coated 100 mm dish.

1.3.1.2. To thaw a vial of hPSCs directly from liquid nitrogen, put the vial into a 37 °C water bath, carefully swinging the tube in the water until it thaws. Transfer the thawed hPSCs to a 15 mL tube containing 10 mL of 1x PBS, and centrifuge at 200 x *g* for 4 min.

1.3.1.3. Discard the supernatant and add 1 mL of E8 medium to the tube. Pipette a few times to fully resuspend the pellet and then add another 9 mL of E8 medium to reach 10 mL total.

1.3.1.4. Aspirate the VTN solution from the 100 mm dish.

1.3.1.5. Transfer the hPSCs to a 100 mm dish, shake gently (up-down and left-right, not in circles) to make sure cells are distributed evenly in the dish

1.3.1.6. Incubate at 37 °C, in 5% CO_2.

1.3.1.7. On the following day, aspirate all medium and feed with 10 mL of E8.

1.3.1.8. Feed this way every day for the next 3–4 days and then prepare to split.

1.3.2. Splitting hPSCs

NOTE: hPSCs at the point of splitting should be 80%–90% confluent. Big colonies with smooth and bright edges should be observed. However, contact between each colony should be avoided (**Figure 2B**, day 0 and **Figure 6B**).

1.3.2.1. Prepare VTN-coated 100 mm dishes as needed.

1.3.2.2. Aspirate the E8 and wash the dish that needs to be split 1x with 1x PBS.

1.3.2.3. Aspirate the 1x PBS and add 4 mL of 0.25 M EDTA. Incubate for 2 min at 37 °C, 5% CO_2.

NOTE: The hPSCs should be split/replated as small colonies. Do not treat with EDTA longer than 2 min to prevent separation into single cells. The cells should be still attached to the dish surface after the 2 min treatment.

1.3.2.4. Aspirate the EDTA, detach the colonies by strongly pipetting 10 mL of E8 medium onto the dish surface, and collect all the medium and cells in a 15 mL tube.

1.3.2.5. With hPSCs at 80%–90% confluency, split colonies by 1:15-1:20. For example, to split hPSCs by 1:20 into one 100 mm dish, take 500 µL of E8/hPSCs solution and mix with 9.5 mL of fresh E8 medium.

1.3.2.6. Plate hPSCs in VTN-coated 100 mm dishes.

NOTE: It is advised to establish the ideal split ratio for each researcher and cell line independently.

1.3.3. Freezing hPSCs

1.3.3.1. For one 100 mm dish of hPSCs that is ready to be split, prepare three cryovials and 3.5 mL of freezing medium.

NOTE: Media and vials should be kept in 4 °C or on ice until usage.

1.3.3.2. Aspirate E8 and wash the dish 2x with 1x PBS.

1.3.3.3. Aspirate 1x PBS and add 4 mL of 0.25 M EDTA, incubate for 2 min at 37 °C, in 5% CO_2.

NOTE: hPSCs should be frozen as small colonies at the time that they would be split. Do not treat the cells with EDTA longer than 2 min to prevent separation into single cells. Cells should be still attached on the dish's surface after the 2 min treatment.

1.3.3.4. Aspirate the EDTA, detach the colonies by strongly pipetting 10 mL of 1x PBS on the dish's surface, and collect all medium and cells in a 50 mL tube.

1.3.3.5. Add 20 mL of 1x PBS and centrifuge at 200 x *g* for 4 min to wash out the remaining EDTA.

1.3.3.6. Discard the supernatant and resuspend the pellet in 3 mL of freezing medium.

1.3.3.7. Distribute hPSCs evenly into the three cryovials, 1 mL each.

1.3.3.8. Store at -80 °C overnight in a controlled freezing box or a styrofoam sandwich to ensure a slow temperature drop, and then transfer to a liquid nitrogen tank for long term storage.

2. Seeding hPSCs to start the differentiation (day 0)

NOTE: hPSCs should be ready for differentiation after being stabilized (i.e., being split 2–3x after thawing). Be sure that the colonies are healthy, with smooth, shiny edges, and minimal differentiation (**Figure 2B**).

2.1. Prepare basement membrane matrix-coated dishes (24 well or 6 well dishes) one day before day 0 as needed. Bring the dishes to RT at the start of differentiation.

2.2. Make the day 0–1 differentiation medium as needed.

2.3. Aspirate the E8 from the confluent, ready to split hPCSs, and wash the dish 2x with 1x PBS.

2.4. Add 7 mL of 0.25 M EDTA per 100 mm dish, incubate at 37 °C, 5% CO_2, for 15 min.

NOTE: At this point, EDTA treatment is prolonged to disperse into single cells.

2.5. Pipet off all the hPSCs (they should be floating) and transfer to a 50 mL tube. Add the same amount or more of 1x PBS as EDTA solution to dilute out the EDTA.

2.6. Centrifuge at 200 x *g* for 4 min.

2.7. Discard the supernatant, add 1 mL of day 0–1 differentiation medium and pipet to

homogenize the cells. Follow by adding more medium and then mix to dilute the cell solution to a concentration ideal to count the cells.

NOTE: Do not overdilute the cell solution. Cells from one full 100 mm dish should be diluted in 5 mL of medium to start.

2.8. Count the cell number using an automated cell counter or hemocytometer.

2.9. Dilute the cell solution as needed to reach 125,000 cells/cm^2 in a low final volume (e.g., 2 mL per well for a 6 well dish or 500 µL per well for a 24 well dish).

NOTE: A low volume helps the cells attach faster.

2.10. Aspirate all of the basement membrane matrix solution from the coated dishes and plate the cell solution into the wells.

2.11. Incubate at 37 °C, in 5% CO_2.

3. Neural crest cell induction (day 1 to day 10, Figure 2A)

3.1. On day 1 feed the cells with day 0–1 differentiation medium (3 mL per well for 6 well dishes and 1 mL per well for 24 well dishes).

3.2. On day 2 feed the cells with day 2–day 10 differentiation medium (3 mL per well for 6 well dishes and 1 mL per well for 24 well dishes).

3.3. From now on, the cells should be fed every other day until day 10 (i.e., the next feeding day will be day 4).

NOTE: From day 6 on, NC ridges should be detected (**Figure 2B**). To check if differentiation is taking place, it is advised to carry a parallel differentiation culture in smaller wells (i.e., 24 wells), that can be stained for SOX10/AP2a and used for marker gene expression along the time of differentiation (**Figure 2B**,**C**).

3.4. If sorting cells, proceed to section 4. Otherwise, proceed to section 5.

4. Fluorescence activated cell sorting (FACS) for neural crest marker CD49D and aggregating NC cells in spheroids

NOTE: For FACS sorting, the samples should be kept on ice and not be exposed to light after staining until sorting.

4.1. Prepare FACS buffer if the cells are sorted.

4.2. Prepare day 10–14 spheroid medium.

4.3. On day 10, remove the medium, and wash 1x with 1x PBS.

4.4. Add dissociation solution (see **Table of Materials**) at 2 mL per well for a 6 well dish or 1 mL per well for a 24 well dish, and incubate at 37 °C, 5% CO_2 for 20 min.

4.5. Pipet off all the hPSCs and transfer to a 50 mL tube.

4.6. Fill up the rest of the tube with FACS buffer and centrifuge at 200 x *g* for 4 min.

NOTE: Each 50 mL tube can accommodate up to 20 mL or less of cell solution. The volume of the FACS buffer should be high enough to neutralize the dissociation solution.

4.7. Discard the supernatant, resuspend the cells with the appropriate amount of FACS buffer (~2 mL per well of a 6 well plate), and count to determine the cell number.

4.8. Prepare the following samples.

4.8.1. Sample 1 (unstained control): 1 x 10^6 cells in 400 µL of FACS buffer. Filter the cells through a 20 µm strainer cap and keep the tube on ice.

4.8.2. Sample 2 (DAPI only control): 1 x 10^6 cells in 400 µL of FACS buffer containing 0.5 ug/mL DAPI. Filter the cells through a FACS tube with a strainer cap and keep the tube on ice.

4.8.3. Sample 3 (CD49d-labeled): Suspend the rest of the cells with FACS buffer containing PE/Cy7-conjugated CD49D antibody (5 µL for 1 x 10^6 cells per 100 µL of FACS buffer) in a 15 mL tube and incubate on ice for 20 min.

4.9. Fill up the tubes with FACS buffer and centrifuge at 200 x *g* for 4 min.

4.10. Discard the supernatant and resuspend every 5–10 x 10^6 cells in 1 mL of FACS buffer containing 0.5 ug/mL DAPI according to the manufacturer's instructions.

4.11. Filter the cells through the FACS tube with the strainer cap and keep the tube on ice.

4.12. Prepare collection FACS tubes containing 2 mL of FACS buffer.

4.13. Sort through the FACS machine with lasers that can detect DAPI and PE-Cy7 to isolate the $CD49D^+$ population.

4.14. After sorting, count the sorted cells.

4.15. Centrifuge all sorted cells and resuspend in day 10–14 spheroid medium to a final

concentration of 0.5 x 10^6 cells per 500 µL of medium.

4.16. Plate 0.5 x 10^6 cells per well into ultra-low attachment 24 well plates.

4.17. Incubate the cells at 37 °C, in 5% CO_2.

5. Aggregating NC cells in spheroids

5.1. If not using FACS to isolate the NC cells and instead aggregating them into spheroids directly, first prepare cells as described in steps 4.2–4.5.

5.2. Fill up the rest of the tube with 1x PBS, and centrifuge at 200 x *g* for 4 min.

5.3. Discard the supernatant, resuspend the cells with an appropriate amount of day 10–14 spheroid medium (e.g., ~2 mL of medium per well for a 6 well plate), and count to determine the cell number.

5.4. Dilute the cells in day 10–14 spheroid medium to 0.5 x 10^6 cells per 500 µL of medium.

5.5. Plate 500 µL of the cell suspension per well in ultra-low attachment 24 well plates.

5.6. Incubate the cells at 37 °C, in 5% CO_2.

6. NC spheroid maintenance and sympathetic progenitor induction (day 10 to day 14, Figure 4A)

6.1. Option 1: Minimal spheroid culture

6.1.1. On day 11, add 500 µL of day 10–14 spheroid medium to the NC spheroids without aspirating existing medium from day 10. Incubate at 37 °C, in 5% CO_2.

6.1.2. On day 12, tilt the plate to accumulate the NC spheroids on one side of the wells. Carefully aspirate and discard as much medium as possible, and feed with 1 mL of day 10–14 spheroid medium.

6.1.3. Keep feeding the cells every day until day 14.

6.1.4. Optional: If the spheroids aggregate and generate a large clump, use a pipette to break the spheroid clumps up. This also ensures that individual spheroids do not get too large.

NOTE: The ideal spheroid size range should be around 100–500 µm. Within that range, the size of individual spheroids is not critical. However, the morphology, such as a smooth and clear edges (**Figure 3** and **Figure 6**) is important for further success. At day 14, each 24 well plate ideally contains about 50–60 spheroids of different sizes within the abovementioned size range.

6.2. Option 2: Expanded spheroid culture

6.2.1. On day 15, to keep NC spheroids, feed with 1.5 mL of day 10–14 spheroid medium containing 0.5 µM RA. Incubate at 37 °C, 5% CO_2.

NOTE: RA should be added fresh for every feeding and always be stored at -80 °C.

6.2.2. From now on, feed every other day up to day 28 and then continue with plating of the spheroids (section 7.1).

NOTE: The growing spheroids are split approximately 1x per week by pipetting them with a 1 mL pipette to break them up. They are split at an approximate ratio of 1:2–1:4. Within the 2 week expansion period, the cells should roughly quadruple in number.

7. SymN differentiation and maturation (Option 1: after day 14; Option 2: after day 28)

7.1. Plating spheroids in regular dishes

7.1.1. Prepare PO/LM/FM-coated 24 well plates.

7.1.2. Prepare symN medium containing 0.125 µM RA (add fresh every feed) and 10 µM Y27632 (day 14 only).

7.1.3. On day 14, tilt the plate to accumulate NC spheroids on one side of the wells. Carefully aspirate and discard as much medium as possible, and feed with 1 mL of symN medium.

7.1.4. Remove LM/FN from the coated plates.

7.1.5. Split and plate each well from the 24 well plate into 4 separate wells of the new, coated 24 well plate. Each original well will have 1 mL, containing ~50–60 spheroids. This yields 250 µL, containing approximately 10–15 spheroids for each well on the new plate.

7.1.6. Add 250 µL of additional medium per well.

NOTE: This is a split of 1:4; make sure that the spheroids are distributed properly within the solution so that the split is relatively even. The number of spheroids is not counted because the final number does not affect the success of generating symNs.

7.1.7. Incubate at 37 °C, 5% CO_2.

7.1.8. On day 15 (or day 29 for option 2), feed by replacing all medium with 1 mL of symN medium containing 0.125 µM RA. From now on, the neurons should be fed every 2 days until day 20 (or day 35 for option 2).

7.1.9. After day 20 (or day 35 for option 2), the neurons should be fed by carefully replacing only half of the existing medium (500 µL). From now on, feed every week unless the medium quickly turns yellow.

7.1.10. Keep feeding weekly until the desired time point.

NOTE: symNs tend to aggregate in ganglia-like structures and are prone to detach from the culture dishes. To prevent this, half-feedings and minimal handling is recommended.

7.2. Plating cells for electrophysiological recording

7.2.1. Prepare PO/LM/FM-coated 96 well electrophysiology plates.

7.2.2. Prepare symN medium containing 0.125 µM RA (add fresh every feed) and 10 µM Y27632 (day 14 only).

7.2.3. On day 14, collect all spheroids, then centrifuge them at 200 x *g* for 4 min.

7.2.4. Discard the supernatant, add 2 mL of dissociation solution, and transfer the mixture back to one of the wells of the ultra-low attachment plate. Incubate at 37 °C, in 5% CO_2 for 20–45 min.

NOTE: Depending on the size of the spheroids, the dissociation period can be longer than 20 min. Check the cell's dissociation every 10 min up to 45 min. Optionally, 0.1 mg/mL of DNase can be added with dissociation solution to prevent free DNA from dead cells making the solution sticky. This is optional in this protocol because the spheroids will not aggregate once they are fully dissociated.

7.2.5. Pipet to fully dissociate the spheroids, then centrifuge at 200 x *g* for 4 min.

7.2.6. Discard the supernatant, resuspend the cells with appropriate amount of symN medium, and count the cell number.

7.2.7. Plate the cells at 100,000/cm^2 in PO/LM/FN-coated electrophysiology wells in 200 µL total volume per well.

7.2.8. On day 15 (or day 29 for option 2), follow the same feeding processes as in section 7.1. The total volume after day 15 (or day 29 for option 2) should be 300 µL per well.

7.2.9. Measure the electrical signals using a multielectrode array machine after day 20 (or day 35 for option 2).

NOTE: In option 2, spheroids can be plated anytime between day 14–day 28. The first electrical signals measurements can be conducted 1 week after plating the spheroids.

REPRESENTATIVE RESULTS:

In this protocol, we give instructions on how to generate symNs from hPSCs. The culture conditions demonstrated here were improved from an earlier published protocol[23,24] (**Figure 1A**) to feeder-free and chemically defined conditions (**Figure 1B**). Two options are provided, one where symNs are made within 20 days, and another where the NCCs can be expanded for 2 weeks to generate more cells that can then be differentiated into symNs (**Figure 1B**, Option 1 and 2).

To properly monitor the symN characteristics of differentiated cells, the PHOX2B-eGFP WA09 reporter line and the parent WA09 PSCs line were employed[19]. All differentiations were conducted in at least three biological repeats, defined as independent differentiation experiments after at least one passage and/or derived from a freshly thawed vial of cells. Differentiation was induced when the confluency of undifferentiated hPSCs reached 80%–90%. The hPSC colonies were round, with shiny, smooth edges and little to no differentiation. The colonies should not touch each other (**Figure 2B**, day 0). Instead of typical dual SMAD inhibition[25], which was previously used for neurectoderm induction, TGF-β inhibition combined with WNT and BMP4 signaling in the first 2 days led to robust expression of the early neural crest marker AP2a. The neural crest marker SOX10 was expressed from day 4 to day 10 (**Figure 2B**). NCCs emerged in dense, darkened ridges visible from day 6 on. These ridges expressed SOX10 (**Figure 2B**, arrows). It was previously shown that SOX10 at the NCC stage correlates with the cell surface marker CD49D[23,26] and thus CD49D can be utilized to sort NC cells that are not reporting any fluorophore from the SOX10 locus. **Figure 2C** shows the expression of NCC marker genes over time. **Figure 2D** indicates that our differentiation efficiency was above 80%. To determine the identity of the remaining cells, qRT-PCR was used to test for contaminating cell types, including BryT-expressing mesoderm, SOX17-expressing endoderm, and PAX6-expressing neuroectoderm; all were found to be absent or expressed at very low levels (data not shown; see **Supplementary Table 1** for all primers). However, some SIX1/EYA1$^+$ placode was detected (data not shown) that may be the source of the remaining cell types. Additionally, it is possible that the remaining cell types are NCC derivatives that are already further differentiated. The HOX code of the NCCs was assessed at this stage (**Figure 2E**). However, at this early stage the HOX signals were very low (note the scale), suggesting that these NCCs were either of cranial-NC character or had not adopted any NCC subtype character yet.

On day 10 the NCCs could be purified using FACS, which yielded around 80% CD49D$^+$ NCCs (**Figure 2D**). An example of a typical gating strategy is indicated in **Figure 2D**. After sorting, the cells were aggregated as NC spheroids (**Figure 3A**). To expand the NCCs and induce trunk-NC-like properties, cells were treated with a combination of FGF2 and CHIR. We tested if sorting for the CD49D$^+$ population yielded better or purer cultures of symNs later on. **Figure 3B** compares unsorted versus CD49D$^+$ sorted versus CD49D$^-$ sorted cell populations. On the left, it can be seen that the positive sorted and the unsorted populations made NC spheroids in a similar fashion, whereas the negative sorted cells did not aggregate properly, did not make round, smooth, healthy looking spheroids and died within 3–4 days. Furthermore, when the NC spheroids were compared at day 14 via qRT-PCR for NC and symN progenitor markers, significant differences between sorted and unsorted cells could not be detected. Noticeably, at this point the expression of sympathetic

progenitor markers was still low and SOX10 levels remained high, suggesting that the spheroids were still composed of cells with NC properties. One day after plating (day 15), both unsorted and CD49D$^+$ spheroids attached well to PO/LM/FN dishes and the neurite outgrowth could be clearly observed (**Figure 3B**, D15 and **Figure 3C**, D29). The unsorted and sorted cultures were carried further to day 35 in parallel, but no major differences were seen (data not shown). Thus, it can be concluded that the sorting step was not essential for the generation of symNs, and therefore it is an optional step in this protocol. However, for less efficient differentiations that do not yield 80% CD49D$^+$ cells, we recommend the sorting procedure.

Next, we tested whether these NC spheroids could be maintained without losing their NC identity (**Figure 3A**, option 2). The NCCs were cultured as spheroids for up to 2 weeks (**Figure 3C**). The morphology of day 28 spheroids and plated cells on day 29 were similar when compared to day 14 and 15 cells in option 1. On day 28, SOX10 expression was maintained at similar levels as day 14. However, the expression of early sympathetic progenitor markers (i.e., ASCL1, PHOX2B, and GATA3) increased (**Figure 3C**, right), suggesting that the extended culture in FGF2, WNT, and RA signaling led to maturation and posteriorization (**Figure 4C** and **F**). Similar effects were previously reported by Kirino et al[21].

After NC expansion (option 1 or option 2), spheroids were plated on PO/LM/FN coated dishes and supplied with multiple neural factors to mature the symNs (**Figure 4A**). On day 20 and 35 (1 week after plating in option 1 and 2, respectively), neurites were observed in a radial pattern extending from the attached spheroids (**Figure 4A**, right). Furthermore, markers related to norepinephrine (NE) synthesis and transportation (i.e., TH, AAAD, DBH) were expressed (**Figure 4B**). The presence of contaminating cell types were examined here again, and expression of ChAT, which can indicate parasympathetic neurons, was found (**Figure 4B**,**E**). However, ChAT is also expressed in cholinergic symNs. Very low levels of VIP, another parasympathetic marker, were detected (data not shown). Furthermore, low levels of BRN3A, ISL1, and RUNX1 were detected (**Figure 4B**,**E**), which could indicate either sensory neurons or trigeminal neurons, i.e. derivatives of placode. Minimal EDNRB (marking enteric neurons) and OLIG2 (marking motorneurons) were detected (data not shown). The identity of the non-neuronal cells remains unclear. However, based on results from our previous symN protocol[23], they were most likely αSMA$^+$ myofibroblasts. Expression of HOX genes was reexamined, and it was found that HOX5–9 were expressed, suggesting a trunk identity. HOX10 (indicative of sacral-NC) was not expressed (**Figure 4C**, **F**). Lastly, mature markers, including nicotinic acetylcholine receptor (CHRNA3/4) and NE-related receptors, including adrenergic receptors (ADRA2A/B2) NE transporter (NET, SCL6A2), and vesicle transporter (VMAT1) were expressed (**Figure 4D**,**G**). No significant difference was found in the expression of these genes in cells that were derived with option 2 as compared to option 1 (**Figure 4E–G**).

We further confirmed our results by performing this protocol on the H9-PHOX2B:GFP reporter line (**Figure 5**). By day 20, the cells formed a dense lawn of neurons and aggregated in clusters of even higher density, indicating a very high differentiation efficiency (**Figure 5A**, top row). The staining showed that most symNs clump in those clusters, which made the assessment and quantification of the overall differentiation efficiency difficult. To highlight staining and

colocalization of specific symN markers, we focused on the less dense areas on the outskirts of the clusters (see yellow box in **Figure 5A**, right). This was also where most contaminating cells were located, so it was not a good area to judge overall efficiency. Nonetheless, typical symN markers; including peripherin (PRPH), ASCL1, TH, PHOX2B, TUJ1 (pan neuronal), DBH, and NET1 (expressed in dots along symN bodies and axons, **Figure 5A,** bottom); were detected. A multielectrode array (MEA) approach was employed to measure electrical activity, and it was found that day 20 symNs fired at about 3–5 spikes per second compared to the negative control of undifferentiated hPSCs (**Figure 5B**). It is important that the cells be evenly distributed in the well in order for each electrode (black dots in **Figure 5B**, bright field) to properly record. Day 20 to day 30 symNs were also stimulated with 1 μM nicotine, which imitates the preganglionic signaling of symNs in vivo and increased the mean firing rate in the cells. Inhibition of the neurons was also measured. β2-adrenergic receptor (ADRB2), located on symN axon terminals, creates a positive feedback loop for NE secretion[29]. Treating the symNs with 1 μM propranolol (a β2-adrenergic receptor antagonist) inhibited their activity (**Figure 5C**, right). These results suggest that the symNs generated by this protocol were functionally active.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic illustration and comparison of symN differentiation protocols. (**A**) Previous protocol by Zeltner et al.[23]. (**B**) Optimized protocol. Option 1 is 20 days long and contains only 4 days of NC spheroid culture. Option 2 is 35 days long and contains a 2 week NC spheroid expansion stage that allows production of more NC cells. VTN = vitronectin, PO = poly-L-ornithine, LM = laminin, FN = fibronectin, SB = SB 431542, CHIR = CHIR 99021, RA = retinoic acid, AA = ascorbic acid, NFs = NGF+BDNF+GDNF.

Figure 2: NC induction. (**A**) Timeline and treatments for NC induction from day 0 to day 10. (**B**) The morphology and formation of NC ridges were monitored every 2 days by bright field microscopy. Cells at each time point after day 2 were co-stained for AP2A (red) and SOX10 (green). All immunofluorescence pictures were counterstained with DAPI. Red arrows indicate the structures of ridges. (**C**) qRT-PCR analysis for the expression profile of NC markers from day 2–10. (**D**) Representative plot of FACS sorting on day 10 for $CD49D^+$ NC populations (left). A typical gating strategy and isotype control is indicated in the first four plots. Quantification of $CD49D^+$ cell percentage on day 10 was also performed. (**E**) qRT-PCR analysis for the expression profile of HOX genes from day 2–10. NC = neural crest. Error bars stem from data of $n \geq 3$ biological repeats, defined as independent differentiation experiments done on separate days from PSC cultures that were at least one split apart.

Figure 3: Neural crest maintenance and expansion. (**A**) Timeline and treatments for NC expansion during day 10 to day 14 culture for option 1 or day 10 to day 28 culture for option 2. (**B**) The NC spheroid formation was monitored by bright field microscopy from day 11 (1 day after spheroid formation) to day 14 (i.e., the day of plating). Unsorted, $CD49D^+$, and $CD49D^-$ populations were compared. The plated cells on day 15 are also shown. Cells failed to form spheroids properly in the $CD49D^-$ group and died after plating. Day 14 cells were examined by qRT-PCR analysis (right) for the expression profile of symN progenitors. Unsorted and $CD49D^+$ populations were compared ($n \geq 3$). (**C**) NC spheroids could be maintained for up to 2 weeks.

Spheroids were monitored by bright field microscopy from day 15 to day 28 (the day of landing). The plated cells on day 29 are also shown. Day 28 spheroids were examined by qRT-PCR analysis for the expression profile of symN progenitors (right). Unsorted and $CD49D^+$ populations were pooled ($n \geq 3$).

Figure 4: SymN differentiation and maturation. (**A**) Timeline and treatments after plating on day 14 for option 1 or day 28 for option 2. Bright field microscopy photos (right) show symNs on day 20 and day 35, respectively (1 week after plating for both options). (**B**–**D**) Option 1 qRT-PCR analysis for the expression profile of symN properties between days 20–30. Unsorted and $CD49D^+$ populations were pooled. (**E**–**G**) Option 2 qRT-PCR analysis for the expression profile of symN properties after day 35. Unsorted and $CD49D^+$ populations were pooled ($n \geq 3$).

Figure 5: Functional characterization of symNs. (**A**, top row) Bright field image of symN clusters at day 20 and stained image showing PRPH and TH double positive cells mostly located in the clusters. (**A**, bottom) Multiple symN markers (separated channels) were verified by immunofluorescence staining on day 20. The norepinephrine transporter (NET1) was located both on the surface of the cell bodies as well as along the axons and dendrites. It showed up as dots at this magnification. All immunofluorescence pictures were counterstained with DAPI. (**B**) Representative heatmaps of multielectrode array (MEA) analysis for symNs on day 20 (top). The bright field picture (bottom) shows the density of symNs and the distribution of electrodes (eight black dots). Unsorted and $CD49D^+$ populations were pooled here. (**C**) Quantification of mean firing rates for day 20–30 symNs under treatments of 1 μM nicotine and 1 μM propranolol for 5 min, respectively (right). Results from unsorted and CD49d-positive populations were pooled ($n \geq 3$). Unpaired, two-tailed t-test with Welch's correction, p-value:*<0.05.

Figure 6: Example of cells under conditions that are inappropriate to proceed with the differentiation. (**A**) Timeline of the differentiation with each check point for cell morphologies indicated as in **B**–**E**. (**B**) hPSCs (a) with healthy colonies and a lot of differentiated cells, and (b) merged and differentiated borders between some colonies on day 0. Red arrows indicate the merged areas. (**C**) NCCs at day 10 with bubble-like blisters in the ridges. Red arrows indicate the blisters in the top row. The bottom row represents a higher magnification. We have not been able to identify the cell identity of the blisters. However, presence of more blisters seems to correlate with lower SOX10/CD49D expression. (**D**) Irregular looking spheroids lacking smooth edges and round shape on day 14. (**E**) Unhealthy and dying symNs on day 20.

DISCUSSION:

We recently published two reviews, one discussing the use of hPSC-derived symNs for disease modeling[31] as well as an in-depth comparison of available differentiation protocols[22]. Thus, here we focus on troubleshooting the current protocol to help the interested researcher succeed in making symNs. During the entire differentiation process, in order to gain consistent data as well as healthy differentiated cells, contamination at all stages should be carefully controlled. In routine hPSC maintenance, mycoplasma testing should be performed biweekly or monthly. If cell sorting is performed on day 10, the addition of antibiotics to any medium is highly encouraged. It is also important to check the cell morphology before and after starting the differentiation.

Here, we suggest several critical check points (**Figure 6A**). First, ideal hPSC colonies ready for differentiation should have bright and smooth edges with tiny spikes. Colonies with gear-like spikes have started to differentiate and should not be used. It is highly recommended that cells at the perfect time point should be used immediately, because the morphology can change within hours. Postponing for just one day may crash the cells (**Figure 6B, a**) or lead to contact between colonies (**Figure 6B**, **b**), which stimulates differentiation in hPSCs. It is challenging to get rid of every differentiated cell in a culture, yet the population should be controlled to contain fewer than 5% of bad colonies. When following this protocol, the second check point during differentiation is between day 2 and day 10. At this stage, dark NC ridges should be clearly observed under a bright field microscope (**Figure 2C**). The thickness and distribution of ridges can be used as indicators of NC efficiency: Because NCCs are mainly derived from cells that form the ridges, thin or less widespread ridges and blisters in the ridges might indicate low NC production, and therefore should be marked or discarded in the case of inconsistent results (**Figure 6C**). RNA sampling is highly recommended on day 10 to check the NC markers described above. The third checkpoint is during the NC spheroid stage, because spheroids may aggregate. It is best to periodically pipet the medium to break up and resuspend the aggregation (cells will not be dispersed back to single cells but small spheroids only). If the spheroids expand too quickly, split them by 1:2 or 1:4 to leave enough space and nutrients for the cells to grow. If spheroids do not have smooth and round shapes (**Figure 6D**), it may indicate that the NC efficiency at day 10 was not high enough, and thus may ruin the final differentiation. Testing expression profiles to check symN progenitor properties is recommended on the desired spheroid plating day. The fourth checkpoint is after plating the spheroids. Neurites should be clearly visible and bundles should start forming after day 20. Cells without widespread neurites and bundles should be considered contamination (**Figure 6E**). At this point, it is important to feed the neurons by replacing only half of the existing medium to maintain the nutritious environment created by stable neurons. Be careful when feeding cells, because maturing symNs are vulnerable and can easily detach. The reason for each of the bad-looking morphologies described here is not completely understood yet. It does, however, provide insight into the sensitive nature of hPSCs and in vitro differentiation. One source of such irregularities might be due to reagents from different vendors. For instance, the thin ridges with blisters show up when using a different brand of BMP4.

For electrophysiological analysis using MEA, in order to evenly distribute symNs in the electrode wells, spheroids are dissociated by Accutase. The treating time should be 20 min to start with. However, because spheroids are highly compacted, the time of dissociation can be increased to 45 min to ensure the spheroids are fully dissociated. In general, each experimental group on a MEA plate needs to be run in multiples of at least six to get consistent results. Notably, because our density for replating is a lot lower than the manufacturer's recommendations[32] (about 500–700,000/cm^2), the well repeats are crucial. In our results, both substantial symN marker expression and stable activity show up from day 20 to day 30. We recommend this as the best time point to start recording the variables of choice. For each differentiation, the medium should be made fresh and used as quickly as possible (no longer than one month).

The symN differentiation protocol presented here is feeder-free, chemically defined, efficient, expandable, and yields functional symNs by day 20. This defined symN differentiation protocol

could be used to study human disorders of the sympathetic nervous system, as a platform for drug screening, for tumorigenesis studies such as neuroblastoma/pheochromocytoma, or for basic research in homeostatic regulation.

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DISCLOSURES:

The authors have nothing to disclose.

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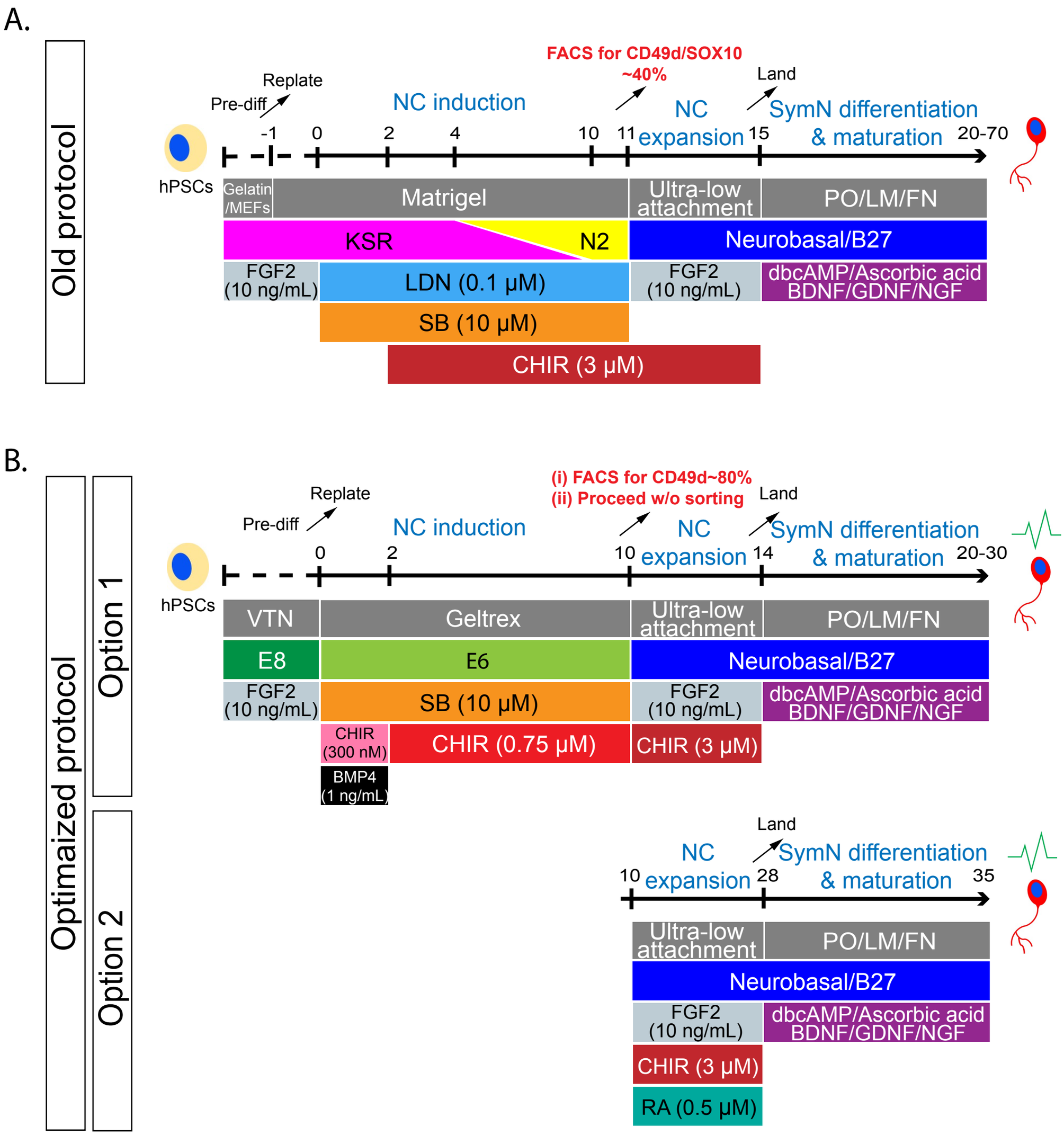

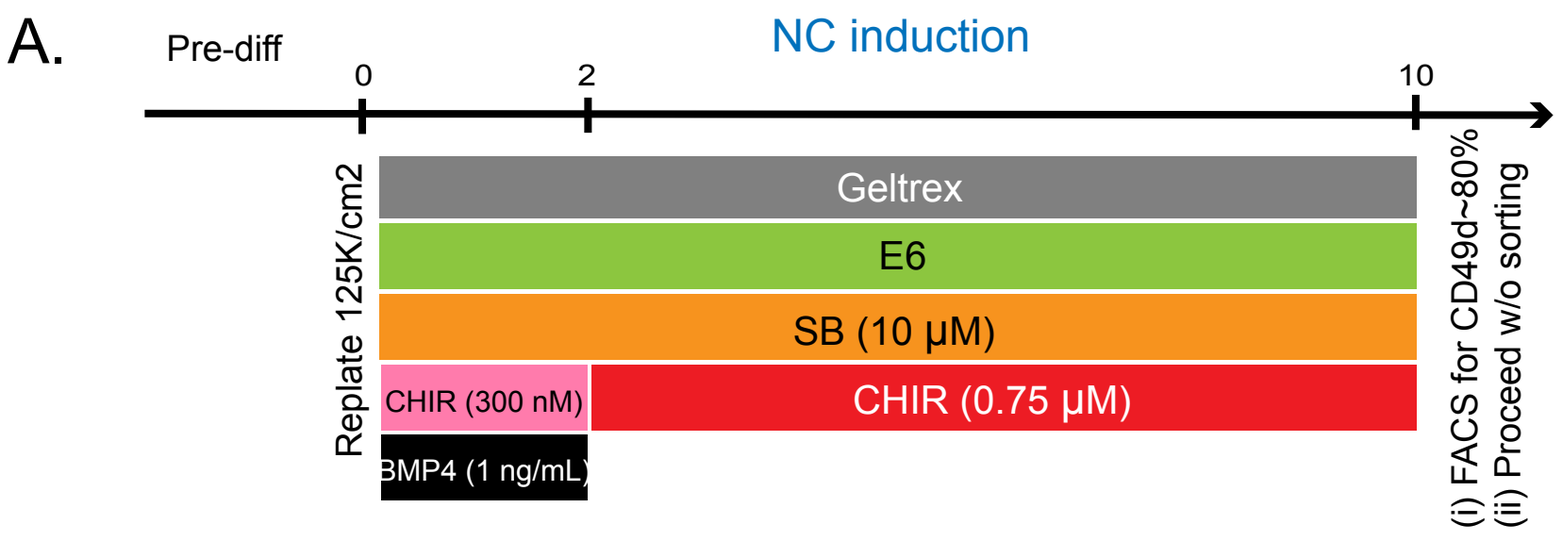

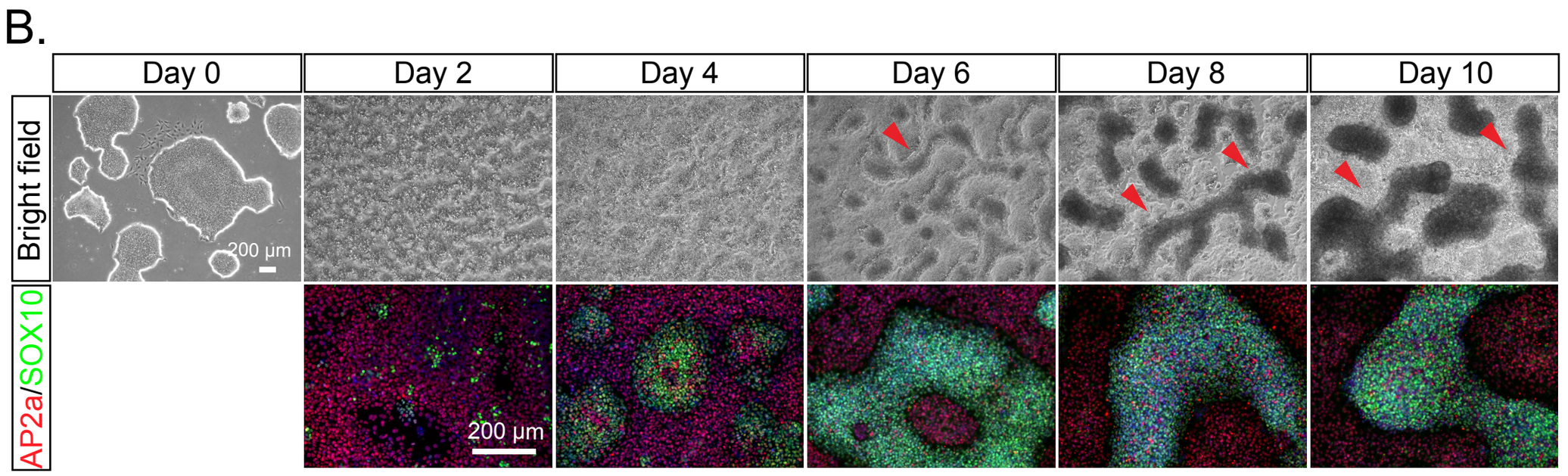

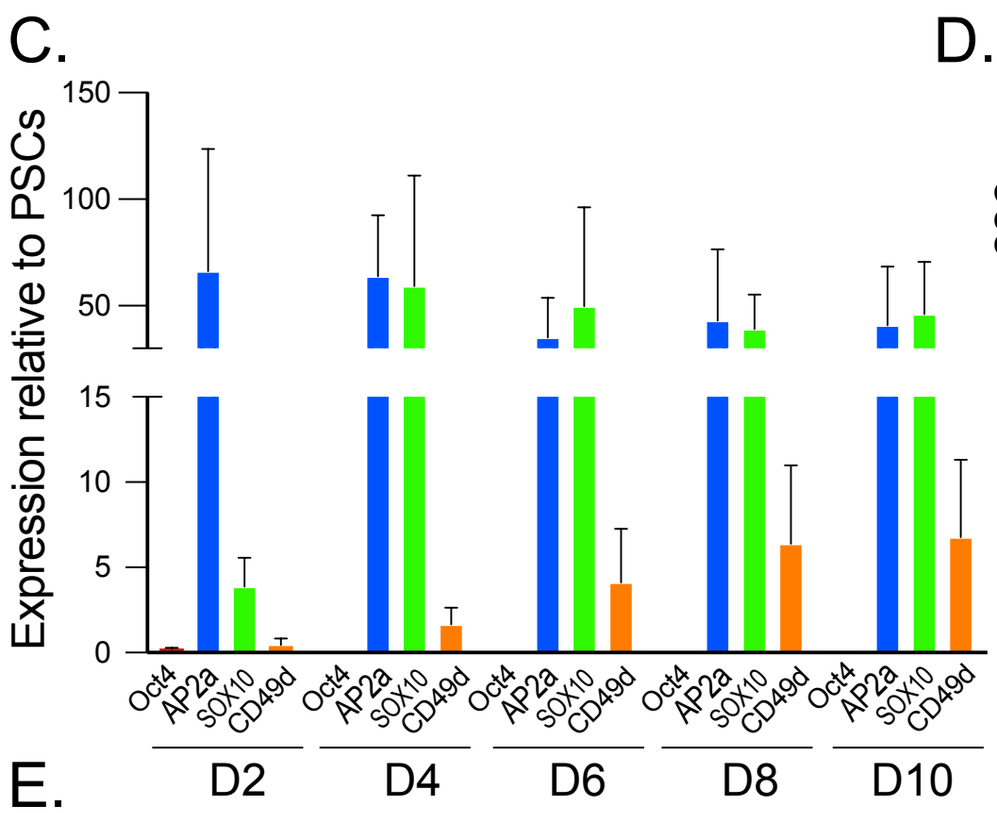

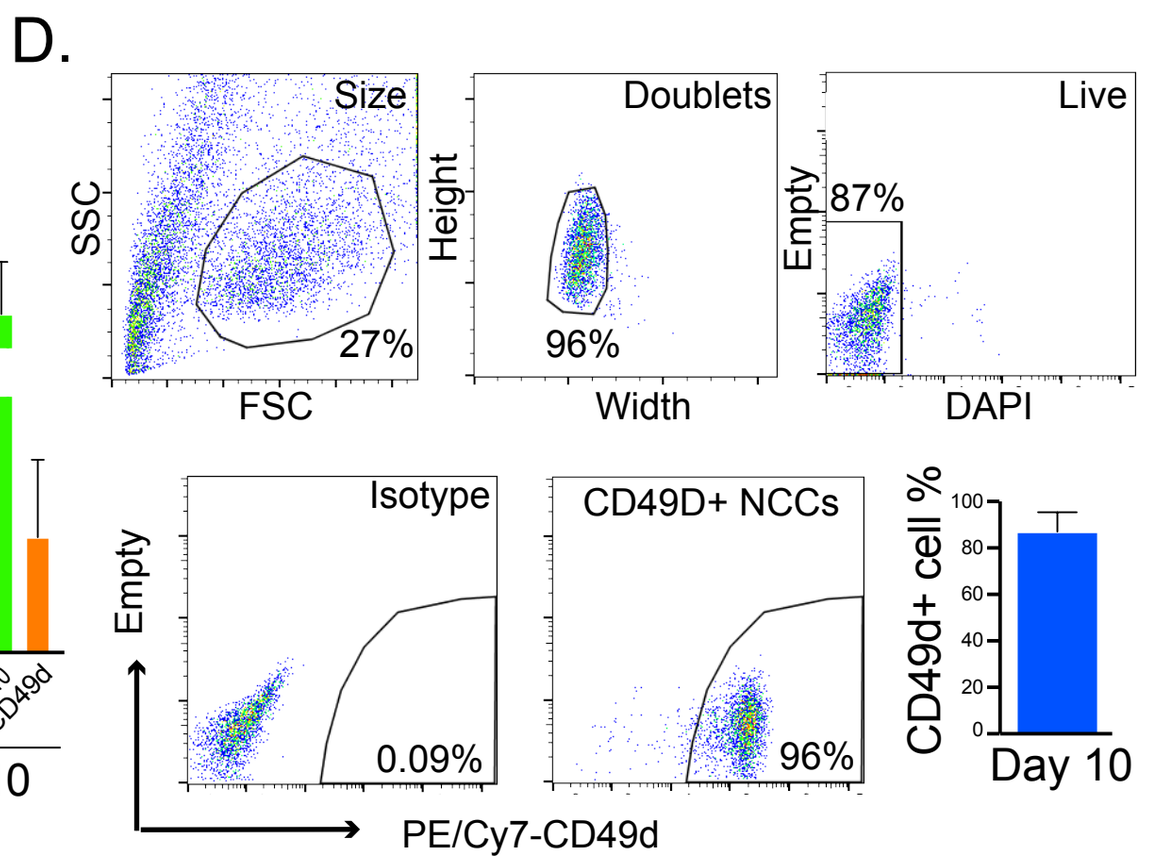

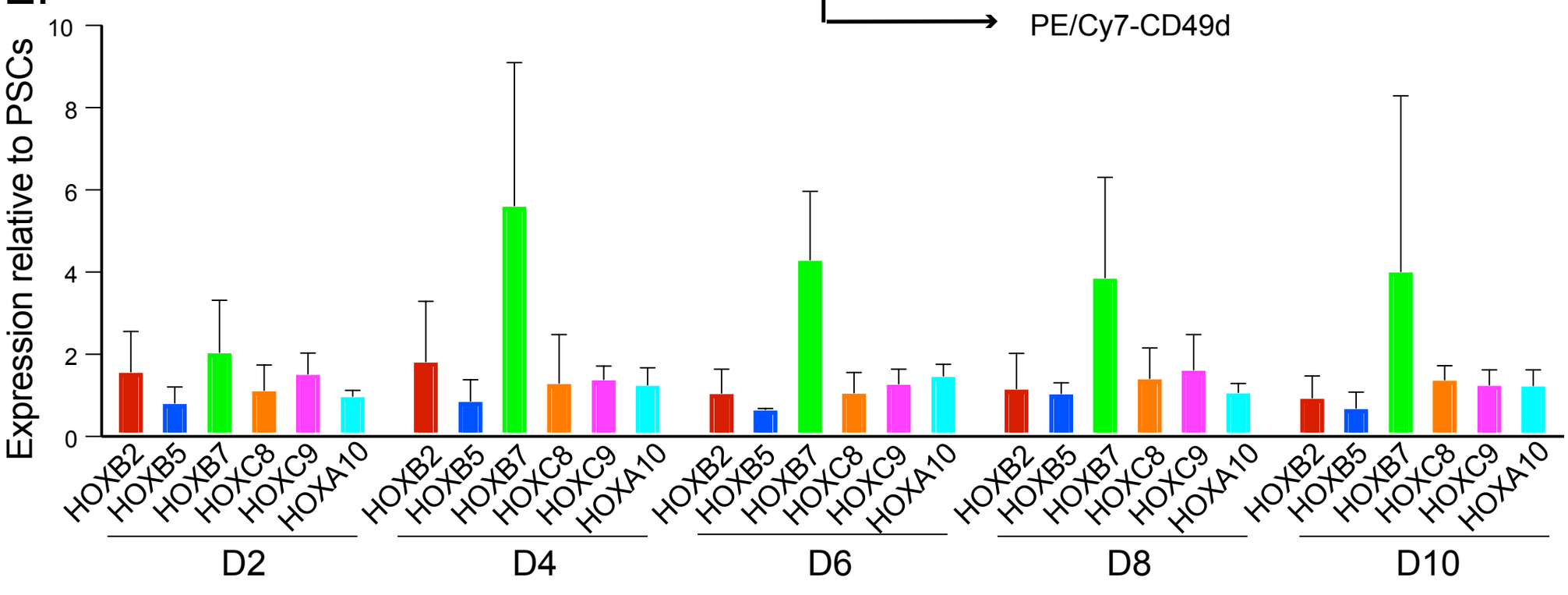

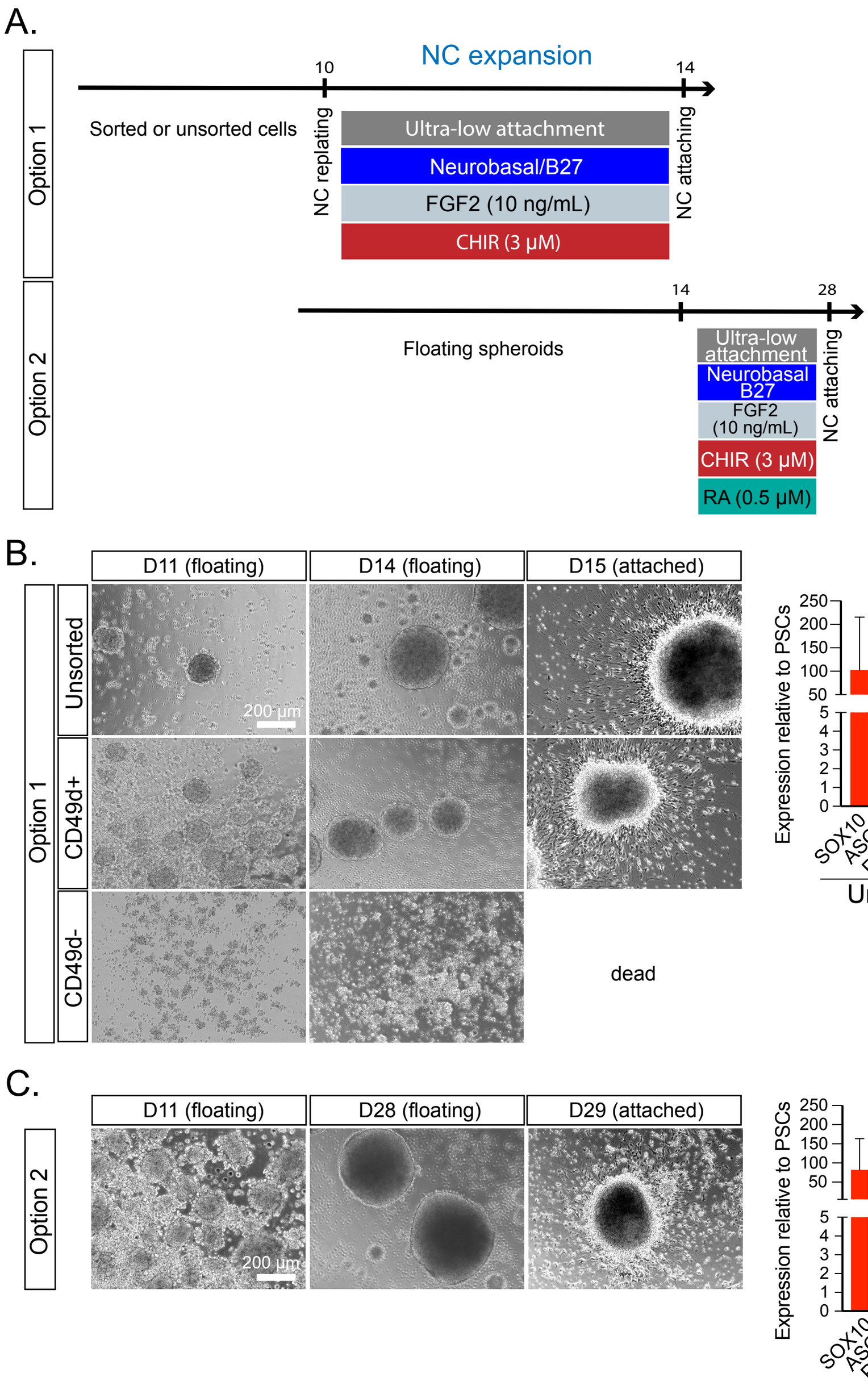

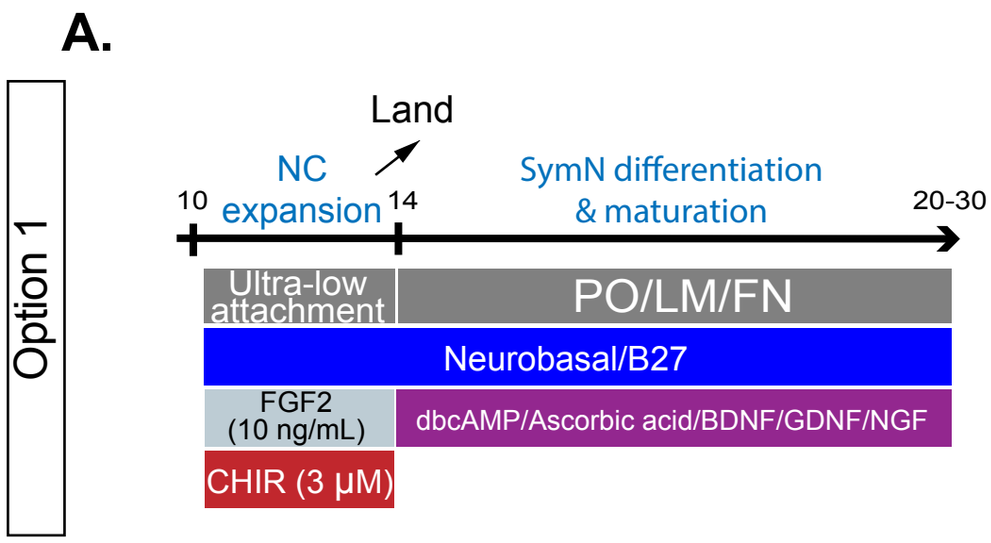

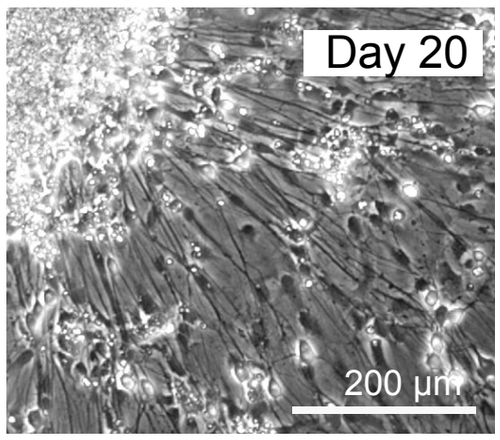

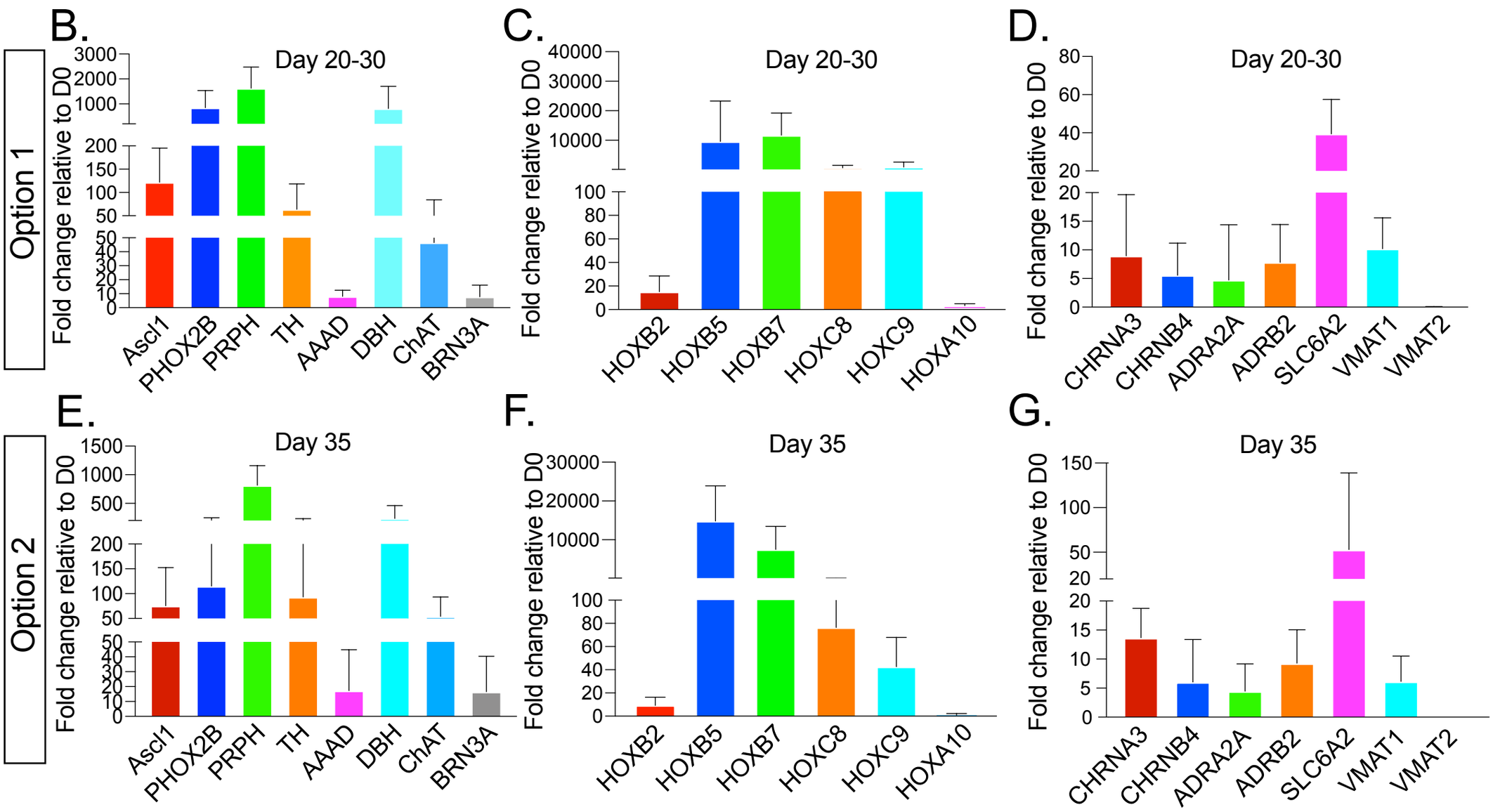

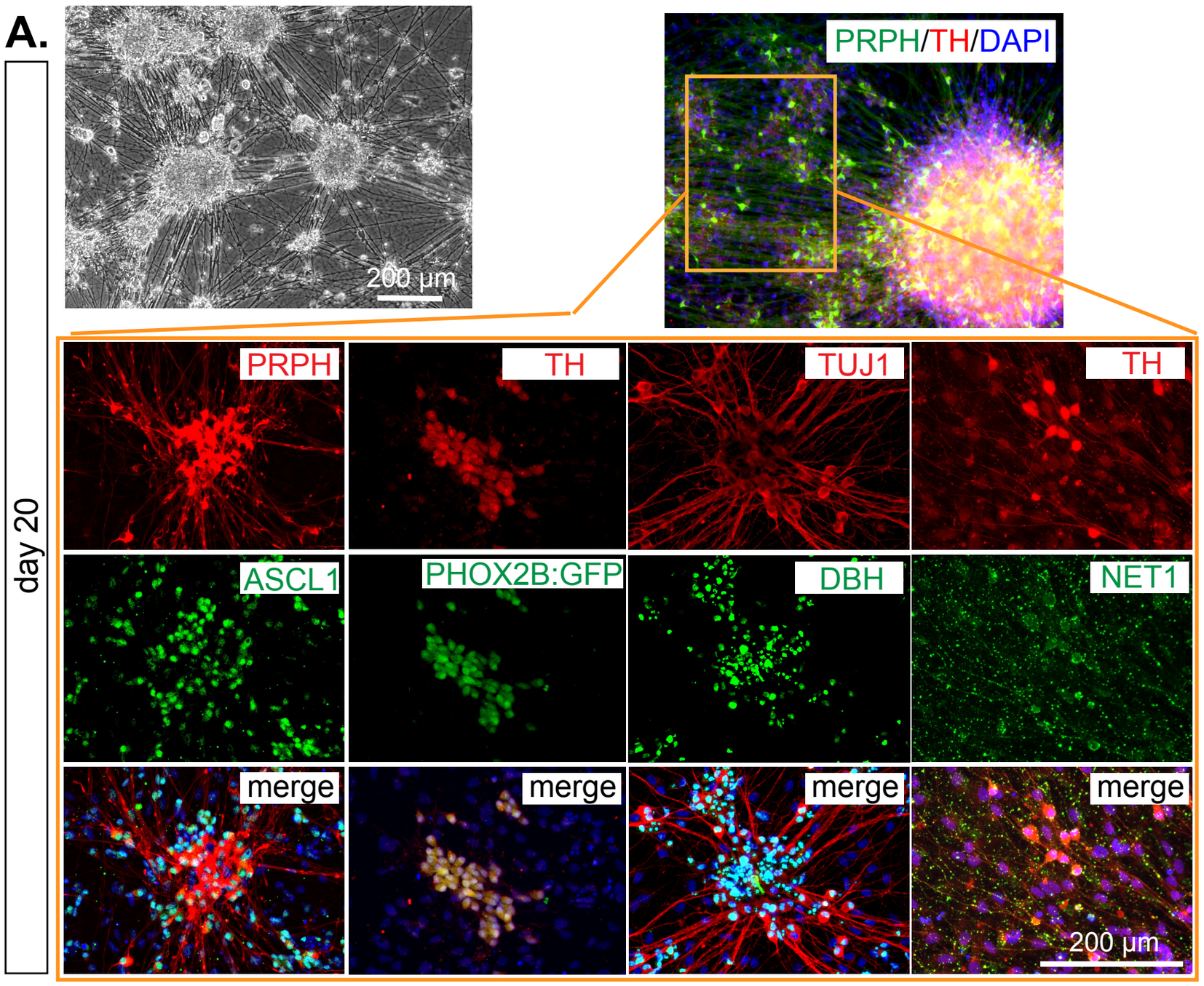

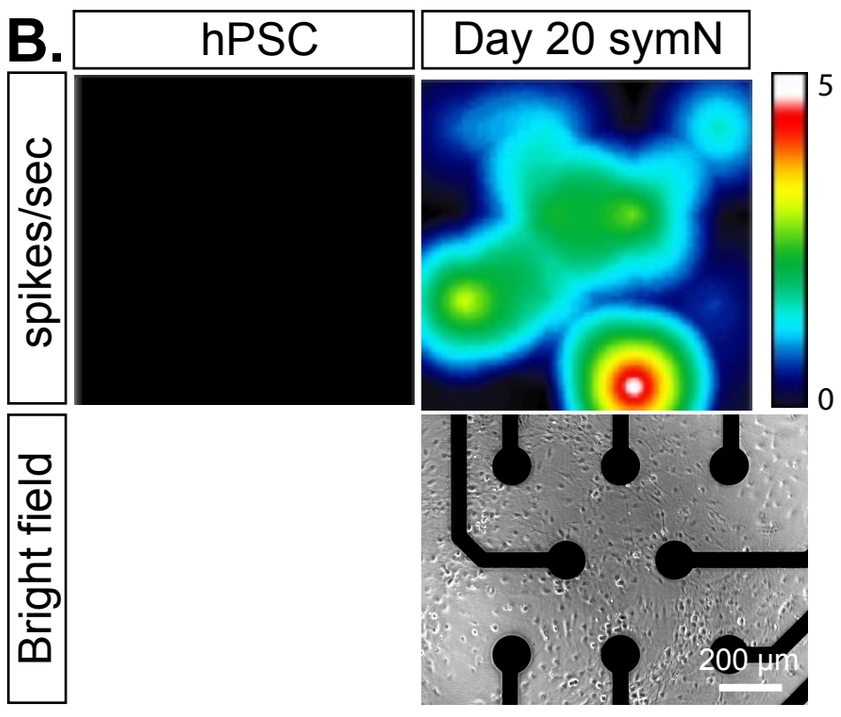

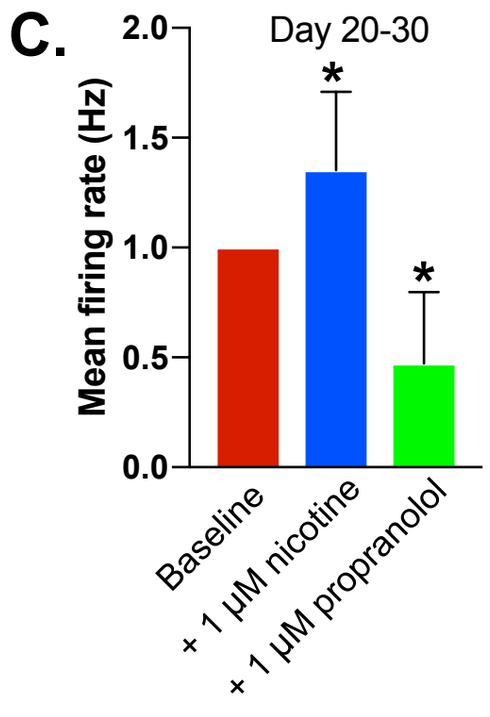

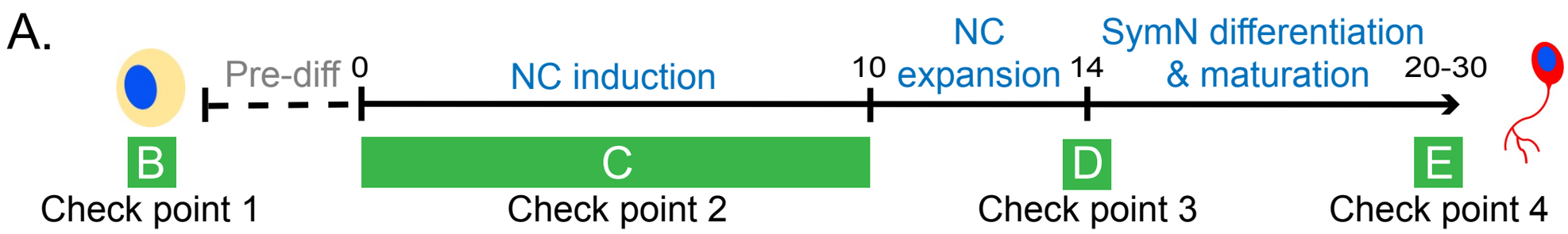

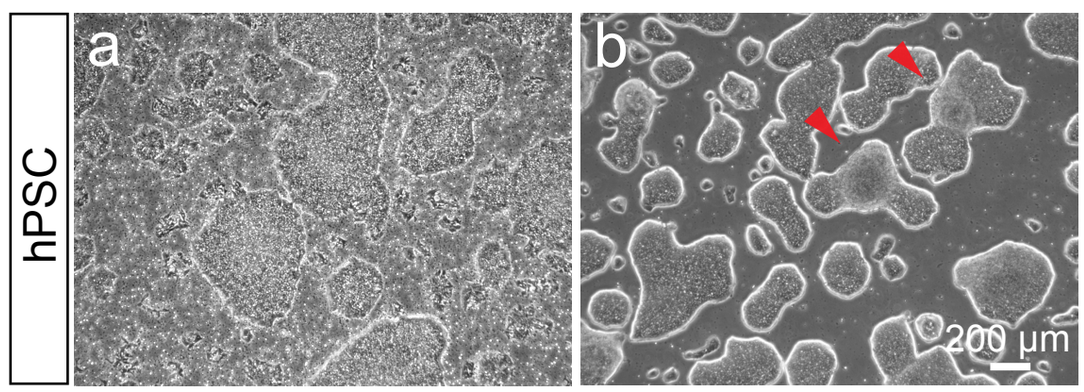

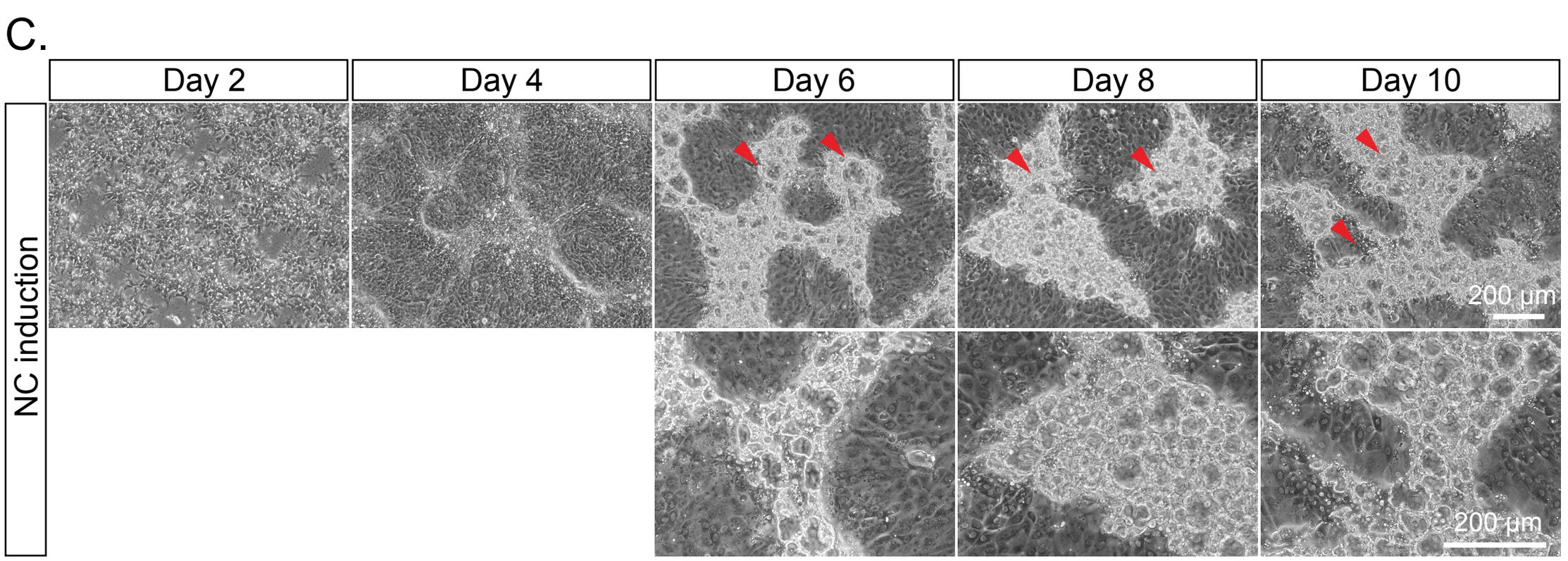

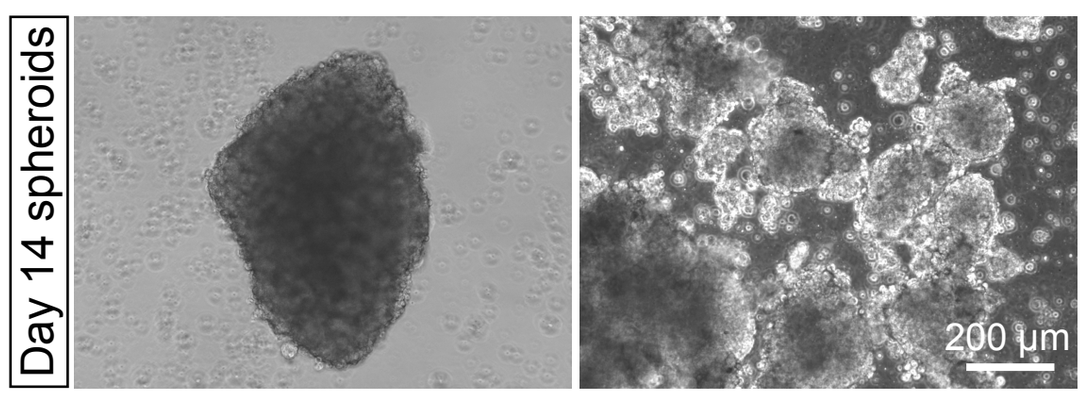

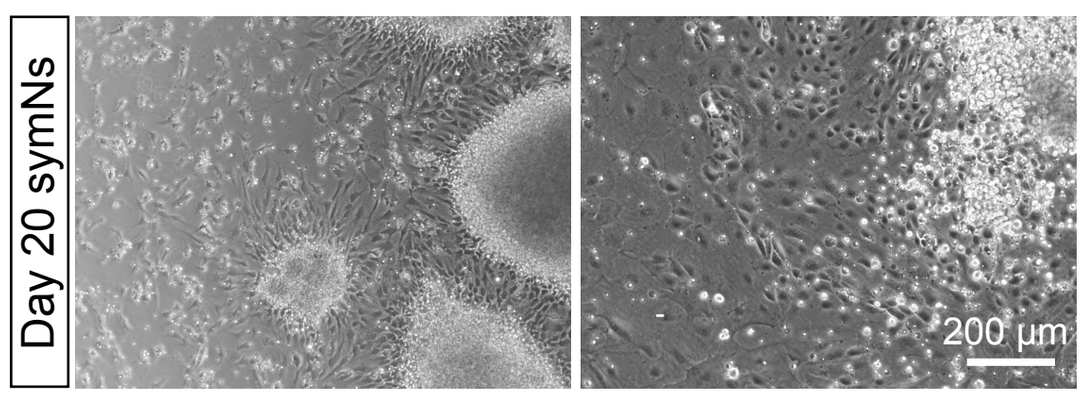

Name of Material/ Equipment	Company
100 mm cell culture dishes	Falcon
15 mL conical tissue culture tubes	VWR/Corning
24-well tissue culture plates	Falcon
24-well ultra-low-attachment plates	Corning
5% CO2/20% O2 tissue culture incubator	Thermo Fisher/Life Technologies
50 ml conical tissue culture tubes	VWR/Corning
6-well tissue culture plates	Costar
Accutase	Innovation Cell Technologies
Anti-AP2a antibody	Abcam
Anti-Ascl1 antibody	BD Pharmingen
Anti-CD49D antibody	BioLegend
Anti-CD49D antibody (isotype)	BioLegend
Anti-DAPI antibody	Sigma
Anti-DBH antibody	Immunostar
Anti-GFP antibody	Abcam
Anti-HOXC9 antibody	Santa Cruz Biotechnology
Anti-NET1 antibody	Mab
Anti-PRPH antibody	Santa Cruz Biotechnology
Anti-SOX10 antibody	Abcam
Anti-TH antibody	Pel-Freez
Ascorbic acid	Sigma
B27 supplement	Thermo Fisher/Life Technologies
BDNF	R&D Systems
BMP4	R&D Systems
Cell counter	Thermo Fisher/Life Technologies
Cell counting chamber slides	Invitrogen
Centrifuge	Eppendorf
CHIR99021	R&D Systems
Cryo-vial	Thermo Fisher/Life Technologies
dbcAMP	Sigma
DMEM	Thermo Fisher/Life Technologies
DMEM/F12	Thermo Fisher/Life Technologies
DMSO	Thermo Fisher/Life Technologies
E6 medium	gibco
E8 medium	gibco
E8 supplement	gibco
EDTA	Sigma
Electrophysiology plates (AXION cytoview MEA96)	Axion BioSystems
FACS machine	Beckman Coulter
FACS machine	Beckman Coulter
FACS tubes (blue filter cap)	Falcon
FACS tubes (white cap)	Falcon

Fetal bovine serum (FBS)	Atlanta Biologicals
GDNF	PeproTech
Geltrex	Invitrogen
hPSCs	Thomson et al., (1998)
hPSCs	Oh et al. (2016)
Human fibronectin (FN)	VWR/Corning
L-glutamine	Thermo Fisher/Gibco
LN tank	Custom Biogenic Systems
MEA reader	Axion BioSystems
Mouse laminin I (LM)	R&D Systems
N2 supplement	Thermo Fisher/Life Technologies
Neurobasal medium	gibco
NGF	PeproTech
Phosphate-buffered saline (PBS)	Gibco
Poly-L-ornithine hydrobromide (PO)	Sigma
Primocin (antibiotics)	InvivoGen
qPCR machine	Bio-Rad Laboratories
qPCR plates	Bio-Rad Laboratories
recombinant FGF2	R&D Systems
Retinoic acid	Sigma
SB431542	Tocris/R&D Systems
Trypan blue	Corning
Vitronectin (VTN)	Thermo Fisher/Life Technologies
Water bath	VWR/Corning
Y27632	R&D Systems

Catalog Number	Comments/Description
353003	
89039-664	
353047	
07 200 601 and 07 200 602	
Heracell VIOS 160i	
89039-656	
3516	
AT104500	Cell dissociation solution
ab108311	Host: Rabbit; 1:400 dilution
556604	Host: Mouse IgG1; 1:200 dilution
304313	Host: Mouse IgG1; 5 µl/million cells in 100 µl volume
400125	Host: Mouse IgG1; 5 µl/million cells in 100 µl volume
D9542	1:1000 dilution
22806	Host: Rabbit; 1:500 dilution
ab13970	Host: Chicken; 1:1000 dilution
sc-365692	Host: Mouse IgG1; 1:100 dilution
NET17-1	Host: Mouse; 1:1000 dilution
SC-377093/H0112	Host: Mouse IgG2a; 1:200 dilution
ab50839	Host: Mouse; 1:100 dilution
P40101- 150	Host: Rabbit; 1:500 dilution
A8960-5G	Stock concentration: 100 mM
12587-010	Stock concentration: 50x
248-BD	Stock concentration: 10 µg/mL
314-BP	Stock concentration: 6 mM
Countess II	
C10312	
57021&5424R	
4423	Stock concentration: 6 mM
375353	
D0627	Stock concentration: 100 mM
10829-018	Stock concentration: 1x
11330-057	Stock concentration: 1x
BP231-100	
A15165-01	
A15169-01	Stock concentration: 1x
A15171-01	Stock concentration: 50x
ED2SS	Stock concentration: 0.5 M
M768-tMEA-96W	
CytoFLEX (for FACS)	
MoFlo Astrios EQ (for sorting)	
352235	
352063	

S11150	
450	Stock concentration: 10 µg/mL
A1413202	Basement membrane matrix; Stock concentration: 100x
WA09	
H9-PHOX2B::eGFP	
47743-654	Stock concentration: 1 mg/mL
25030-081	Stock concentration: 200 mM
V-1500AB	
Maestro Pro	
3400-010-01	Stock concentration: 1 mg/mL
17502-048	Stock concentration: 100x
21103-049	Stock concentration: 1x
450-01	Stock concentration: 25 µg/mL
14190-136	Stock concentration: 1x
P3655	Stock concentration: 15 mg/mL
ANTPM1	Stock concentration: 50 mg/mL
C1000 Touch	
HSP9601	
233-FB/CF	Stock concentration: 10 µg/mL
R2625	Stock concentration: 1 mM
1614	Stock concentration: 10 mM
MT-25-900-CI	
A14700	Stock concentration: 0.5 mg/mL
706308	
1254	Stock concentration: 10 mM

Rebuttal Wu et al.

Dear editors and reviewers,
Thank you for the opportunity to review and your help to improve our manuscript. We have addressed each comment/request in the text below (highlighted in green and indicated with the line number). If we made corresponding changes in the manuscript, those changes are also highlighted in green.

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps. Done.
3. Please include at least 6 key words or phrases. Done.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Geltrex, Accutase, AXION Maestro Pro MEA. We have replaced all mention of AXION Maestro Pro MEA in the manuscript. However, we are not sure how to call Geltrex or Accutase otherwise without causing major confusion for the reader.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps. Done.

Figures:
1. Please insure there are spaces between numbers and their corresponding units. Please use 'µ' instead of 'u', and 'L' instead of 'l' for volumes. Done.
2. Please remove 'Figure 1' etc. from the figures themselves. Done.
3. Figures 2 and 3: 'Fold change relative to D0' looks wrong. Done.

References:
1. Please do not abbreviate journal titles. Done.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol. Done.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors present a thorough and well-presented protocol for the generation and characterization of human pluripotent stem cell (hPSC) derived sympathetic neurons. Given the range of applications from disease modelling and drug discovery that is possible with efficient protocols to derive these cells, there is a need for manuscripts that describe the generation of these cell types in vitro to facilitate their use.
They give detailed protocols & checkpoints to guide users through the protocol from the initial growing of hPSCs all the way through the differentiation to allow for this protocol to be followed with ease and without the requirement of much more information.
The authors also provide a protocol with which to characterize the physiological characteristics of hPSC derived sympathetic neurons on multi-electrode array plates with sample data for end users to compare their findings to.
Furthermore, they provide checkpoints at all stages of differentiation, that are well documented in the figures which can allow for users to identify the success of the differentiation as they proceed.

Minor Concerns:
-some minor typographical errors
- For section 4 regarding Fluoresence Activated Cell sorting (FACs), the negative control for CD49d immunoreactivity suggested is unstained cells. An appropriate isotype control should be recommended to set a the fluoresence threshold. There is a related isotype control (Biolegend Cat #400125). We agree that this is a missing point in our results and we have now added this control in **Figure 2D**. Additionally, the CD49D staining and FACS isolation of neural crest cells has been established and reported previously (Fattahi, 2016, Nature and Zeltner 2016, Nature Medicine. We now discuss this better in the manuscript (line 435).

- A gating strategy for FACs would be useful. It is not clear what plots and gates have been used to select for the appropriate cell size or live dead exclusion gates look like that give plots similar to that from Figure 2D. These data would greatly enhance reproducibility. We have adjusted **Figure 2D** now to show the representative FACS gating strategy that we used, including FSC/FSS for cell size, width-hight for doublet exclusion, DAPI and isotype control antibody gates (Line 449 and Figure legend, Line 525).

-For section 5- spheroid maintenance of further details on the size of the spheroids that are generated should be given. In particular, what size spheroids are desired for the

downstream derivation of sympathetic neurons. As we mentioned in the discussion, spheroids sometimes aggregate over time. To prevent the generation of a big aggregation of spheroids, we recommend to periodically pipet the spheroid solution in order to break these aggregations apart (line 350, step 5.1, substep 4). We now also provide a proper size range of the spheroids (line 353) in the manuscript and discuss the importance of the spheroid appearance as well.

-Related to this, for section 6.1 (5)- a guide to the number of spheroids landed for optimal derivation of sympathetic neurons is not clear. i.e 2 per well of a 24 well plate. We have now added more description in the manuscript to clarify this point (line 356).

Reviewer #2:

Manuscript Summary:

This manuscript by Hsueh Fu Wu & Nadja Zeltner describes a novel feeder-free protocol to differentiate postganglionic sympathetic neurons from hPSC in chemically defined culture conditions. The protocol is well explained and it is very clear. I appreciate that the authors compare the results obtained with this new improved protocol vs their own previous protocol and mentioned why this new protocol is more efficient. I have some minor edits.

Major Concerns:

None

Minor Concerns:

Line 207/208/211

Is it 0.05% trypsin-EDTA or just EDTA? Because both can be used to detach hPSC, it is confusing if the authors first called it 0.05% trypsin-EDTA and then just EDTA.

Same for line 226, 228, 230, 232

It is 0.25 M EDTA, without trypsin. That has been adjusted throughout the text.

Line 240:

Be shure please correct to Be sure. Done.

Line 247:

Is this EDTA or 0.05% trypsin-EDTA?

It is 0.25 M EDTA, without trypsin.

Line 327 and other places:

Can you please add (when it is needed) incubate at 37C, 5% CO2 incubator. Done.

Line 340: do you need to add DNase or it is not necessary? DNase is not necessary in this protocol, we have added a note to clarify this now (line 401).

Fig 5A, Net 1 staining: what are all those dots? We have now explained in the text (line 506) and figure legend that the norepinephrine transporter (NET1) is located both on the surface of the cell bodies as well as along the axons and dendrites, which shows up as dots in staining of this magnification.

Representative results:
No fig 6? Figure 6 is addressed in the discussion section. We have now clarified this in the text (Line 599).

Line 490: 2 full stops. Also optained vs obtained. Both are corrected now.

Line 531
Differnetiations vs differentiations. Corrected.

Catelog/model vs catalog Corrected in Table.

Reviewer #3:
Wu et al. described a comprehensive protocol to derive sympathetic neurons from human pluripotent stem cells (PSCs) under a defined condition. The novelty is of the protocol is little, because it seems just following the previous reports describing derivation of sympathetic neurons from PSCs under defined conditions. However, the protocol is well written and easy to follow up. I just have two comments.

1) How is the differentiation efficiency of sympathetic neurons from neural crest cells? PHOX2B, TH or DBH positive cells seems less than 50% (Figure 5). Please quantify. We do agree with reviewer 3 that quantification of the symN markers would be highly beneficial to claim the exact differentiation efficiency. However, due to the nature of the symNs to clump it is difficult to estimate or quantify the exact efficiency. We have now adjusted **Figure 5** to better represent this (Line 499). On the periphery of the clumps, where one can get beautiful staining and co-staining the efficiency is about 50%, however we do not believe that this is an adequate representation of the efficiency of the entire differentiation. Additional data such as intracellular FACS and FACS on the PHOX2B:GFP reporter line may answer this question more definitively, however we were not able to perform these experiments adequately within this review period and do not think that this is critical for the current protocol.
Additionally, we have now included some more data addressing the identity of contaminating cells in our differentiation, both early on at the NC stage (line 442) as well as at the neuronal stage (line 480).

2) Two clones with PHOX2B reporter seems not sufficient to prove the robustness of the protocol. At least 3 or more clones are required, and efficiency and yields should be

described for each clone. We are not csure that we understand this concern correctly. We assume reviewer 3 is referring to WA09 (i.e. H9) and PHOX2B:GFP reporter as clones. All experiments shown here were performed with these two hPSC lines. WA09 hPSC lines were obtained from WiCell and are not sold as varying clones, there is only one clone available. Similarly, we obtained the PHOX2B:GFP reporter line (which is a modified WA09 line) from the Lee lab (Oh et al., 2016) and were not supplied with multiple clones. Thus, all experiments in this report were conducted in two hPSC lines, both based on WA09. The figures represent 3-10 biological repeats, which are defined as individual differentiation experiments conducted on different days from hPSC cells that have different passage numbers. We have now clarified this point in the text (Line 427). Additionally, we have employed this protocol on various iPSC lines (and clones), both healthy controls and Familial Dysautnomia patient-derived (data not shown or discussed here). The extensive use of our 'old' protocol with these iPSC lines was published (Zeltner, 2016, Nature Medicine). We do not think that the current protocol's scope is appropriate to address the data we obtained with our 'new' protocol and the iPSC lines and thus this is not included here.

Response to 2nd editorial comments.

Changes that were made to the manuscript text are highlighted in blue font now.

1. Please change the citations back to numbered superscripts, with references numbered in order of appearance. Additionally, several references are repeated (Kirino 2018, Oh 2016, Saito-Diaz 2019). Done.

2. There are still a few typos in the figures, e.g.-
Figure 1: '10 ng/ml' (should be 'mL'), '3μM' (include a space). Corrected.

Figure 2: '200μm', 'Hight'. Corrected.

3. Please define the error bars in Figures 2-4. Defined in figure legends now.

Primers	Catalog/Reference	Method	Sequence
AAAD	Origene (NM_000790)	SYBR	Forward
			Reverse
ADRA2A	Origene (NM_000681)	SYBR	Forward
			Reverse
ADRB2	Origene (NM_000024)	SYBR	Forward
			Reverse
AP2a	Origene (NM_003220)	SYBR	Forward
			Reverse
Ascl1	Origene (NM_004316)	SYBR	Forward
			Reverse
BRN3A	Origene (NM_006237)	SYBR	Forward
			Reverse
CD49d	Origene (NM_000885)	SYBR	Forward
			Reverse
ChAT	Home-made	SYBR	Forward
			Reverse
CHRNA3	Origene (NM_000743)	SYBR	Forward
			Reverse
CHRNB4	Origene (NM_000750)	SYBR	Forward
			Reverse
DBH	Origene (NM_000787)	SYBR	Forward
			Reverse
GAPDH	Origene (NM_002046)	SYBR	Forward
			Reverse
GATA3	Origene (NM_001002295)	SYBR	Forward
			Reverse
GR_NR3C1	Origene (NM_000176)	SYBR	Forward
			Reverse
HOXB2	Home-made	SYBR	Forward
			Reverse
HOXB5	Home-made	SYBR	Forward
			Reverse
HOXB7	Home-made	SYBR	Forward
			Reverse
HOXC8	Home-made	SYBR	Forward
			Reverse
HOXC9	Frith et al. (2018)	SYBR	Forward
			Reverse
HOXA10	Home-made	SYBR	Forward
			Reverse
HAND2	Origene (NM_02197)	SYBR	Forward
			Reverse

MAOA	Origene (NM_000240)	SYBR	Forward
			Reverse
Oct4	Origene (NM_002701)	SYBR	Forward
			Reverse
PHOX2B	Frith et al. (2018)	SYBR	Forward
			Reverse
PRPH	Home-made	SYBR	Forward
			Reverse
SLC6A2	Origene (NM_001043)	SYBR	Forward
			Reverse
SOX10	Home-made	SYBR	Forward
			Reverse
TH	Origene (NM_199292)	SYBR	Forward
			Reverse
VIP	Origene (NM_003381)	SYBR	Forward
			Reverse
VMAT1	Origene (NM_003053)	SYBR	Forward
			Reverse
VMAT2	Origene (NM_003054)	SYBR	Forward
			Reverse

GGACCACAACATGCTGCTCCTT
CTCCACTCCATTCAGAAGGTGC
CTTCTGGTTCGGCTACTGCAAC
GGAAACCTCACACGATCCGCTT
TACCAGAGCCTGCTGACCAAGA
AGTCACAGCAGGTCTCATTGGC
GACCTCTCGATCCACTCCTTAC
GAGACGGCATTGCTGTTGGACT
TCTCATCCTACTCGTCGGACGA
CTGCTTCCAAAGTCCATTCGCAC
AGTACCCGTCGCTGCACTCCA
TTGCCCTGGGACACGGCGATG
GCATACAGGTGTCCAGCAGAGA
AGGACCAAGGTGGTAAGCAGCT
GCCTTCTACAGGCTCCATCG
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CGCCCTTTGAGCAGAACCTC
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CGGCCTTTGGTTTTCTTGTCGTT

TCTGAGCCTCACGAAGTGTCTG
ATCCGTTCGCTCACTTGACCAG
CCTGAAGCAGAAGAGGATCACC
AAAGCGGCAGATGGTCGTTTGG
CTACCCCGACATCTACACTCG
CTCCTGCTTGCGAAACTTG
GTGCCCGTCCATTCTTTTGC
GTCACCACCTCCCCATTCCG
CAGGTTCAGCAACGACATCCAG
GTCGTAGGTGAGTGGCTTGAAG
CCAGGCCCACTACAAGAGC
CTCTGGCCTGAGGGGTGC
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CCTGTACTGGAAGGCGATCTCA
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CAGCCTTCCAAAGTCTCTCCTG
GCACATGGTCTGCATCATCCAG
GCTATGCCTTCCTGCTGATTGC
CCAAGGCGATTCCCATGACGTT