Neural differentiation of PSC spheroids grown in StemScale medium

Overview

The following suspension culture protocol provides guidance for generating and expanding neural stem cells (NSCs) starting from pluripotent stem cell (PSC) spheroids cultured in Gibco™ StemScale™ PSC Suspension Medium. During differentiation, PSC spheroids are first induced to NSCs with Gibco™ PSC Neural Induction Medium and cultured as cell aggregates (Figure 1). Next, the NSC aggregates are expanded in Neural Expansion Medium. Finally, the aggregates are dissociated and differentiated in monolayer culture to mature neurons in Neuronal Maturation Medium. Protocols are provided for performing suspension culture neural differentiation in both 6-well format and large-scale 500 mL flasks. The 6-well plate format is ideal for optimizing suspension culture differentiation before scaling up to the 500 mL flasks.

Notes: PSCs for this procedure should be adapted to StemScale medium (at least 3 passages) and can be cultured like any other PSC line following the protocol for StemScale medium (see the StemScale PSC Suspension Medium user guide).

This protocol was optimized using the Thermo Scientific™ CO_2 Resistant Shaker (Cat. No. 88881101). Different shakers may need additional optimization of agitation speed.

See the appendix for media preparation, additional guidance, and supplemental figures.

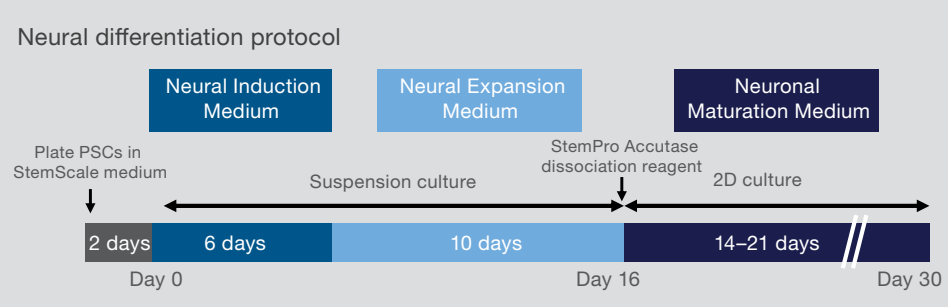

Figure 1. Outline of neural differentiation protocol using spheroids grown in StemScale medium.

Neural induction and expansion of PSC spheroids, 6-well plate format

Neural induction in a 6-well plate

The first step of the neural differentiation protocol is neural induction of spheroids (grown in StemScale medium) to NSC aggregates. After 6 days of neural induction, the resulting NSC aggregates contain >90% SOX1/nestin co-positive cells. Positional marker expression is typically ~30–50% PAX6-positive cells and <5% Otx2-positive cells. See the appendix for supplemental figures showing NSC aggregate morphology during induction and expansion (Figure S1), staining of NSC and positional markers (Figure S2), and staining of neuronal maturation markers (Figure S3).

1. After passaging, maintain PSC spheroids in 2 mL of StemScale medium for 2 days. Average spheroid diameter at the time of induction should be between ~200 µm and 280 µm. See Figure 2 for an example of PSC spheroids at day 0 of induction.

   Note: Spheroid size at the start of differentiation and/or the length of time maintained as spheroids (2 days or 3 days) may need to be optimized depending on cell line. PSC spheroid size can also be controlled by adjusting the shaker rotation speed (higher rpm will result in smaller spheroids).

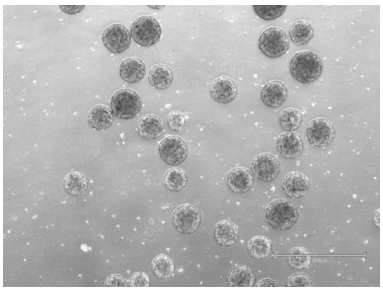

Figure 2. Neural induction at day 0. PSC spheroids grown in StemScale medium after 2 days in suspension culture.

2. Induction day 0 (D0): Perform a 90% medium change, replacing 1.8 mL of StemScale medium with PSC Neural Induction Medium. Return cells to the orbital shaker at 70 rpm. Incubate cells for 48 hr.

3. Induction D2: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 70 rpm. Incubate cells for 24 hr.

4. Induction D3: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 70 rpm. Incubate cells for 24 hr.

5. Induction D4: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 70 rpm. Incubate cells for 24 hr.

6. Induction D5: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 70 rpm. Incubate cells for 24 hr (see Note below).

7. Induction D6: At day 6 of induction (Figure 3), cells should be >90% SOX1/nestin co-positive and are ready to go into Neural Expansion Medium.

Note: Due to proliferation of cells during induction, aggregates may need to be divided into multiple wells between day 5 and day 7 of the protocol. Monitor the medium daily; if the medium is yellow 24 hr after a medium change, divide the aggregates from a single well into multiple wells (aggregate dissociation is not recommended at this stage). A 1:3 well distribution is recommended. The distribution ratio should not be too high, as the agitation dynamics can change and damage the aggregates. See the appendix for guidance on well distribution of NSC aggregates.

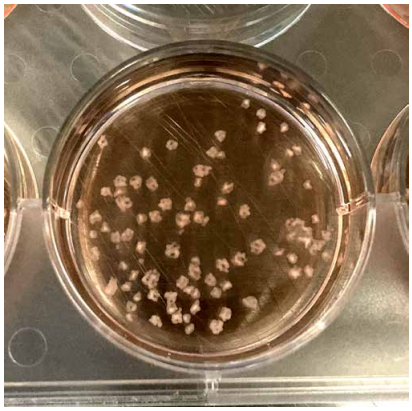

Figure 3. Neural induction at day 6. PSC spheroids in a 6-well plate in suspension at day 6 of neural induction.

Neural expansion in a 6-well plate

After 6 days of induction, NSC aggregates are ready for neural expansion. During expansion, it is normal for larger cell aggregates to partially disaggregate into smaller cell aggregates (Figure S1).

1. Expansion D0: Perform an 80% medium change with Neural Expansion Medium. Return cells to the orbital shaker at 70 rpm for 24 hr. If NSC aggregates have not yet been distributed, follow the guidance on well distribution of NSC aggregates in the appendix, using Neural Expansion Medium.

   Recommended: Neural induction efficiency can be assayed anytime during expansion. Aggregates can be dissociated using Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent and plated in 2D on plates coated with Gibco™ Geltrex™ matrix, for immunocytochemistry (ICC) analysis of NSC markers SOX1/nestin, and positional markers PAX6/Otx2. See the appendix for guidance on dissociating NSC aggregates and 2D plating for induction efficiency assays, and Figure S2 for expected results.

2. Expansion D1 to D8: Perform an 80% medium change with Neural Expansion Medium. Return cells to the orbital shaker at 70 rpm. Perform an 80% medium change daily between D2 and D8. Wells containing aggregates will likely need to be divided 1:3 again between expansion D2 and D4.

3. Expansion D8: At day 8 of expansion, aggregates can be dissociated and plated in 2D for neuronal maturation, or cryopreserved in Neural Expansion Medium with 10% DMSO. See the neuronal maturation section and the appendix for guidance on dissociating and plating NSCs for 2D neuronal maturation. Aggregates can be expanded for at least 10 days without a significant drop in NSC marker expression or impact on subsequent neuronal maturation.

   Note: Between D6 and D8 of expansion, NSC aggregates may need to be divided a third time. Monitor consumption of the medium.

4. Expansion D10: We recommend plating cells for maturation in 2D culture and/or cryopreserving cells.

Neural induction and expansion of PSC spheroids, 500 mL flask format

The large-scale neural differentiation format has been optimized to follow the same timeline as the 6-well plate format, using a 500 mL flask with a culture volume of 100 mL on the Thermo Scientific CO_2 Resistant Shaker at a speed of 80 rpm throughout the protocol.

Note: To visually monitor cell aggregates under a microscope during culture, tilt the flask at a 45° angle and allow aggregates to settle for ~1 min. With a 5 mL pipette, transfer a small number of aggregates to a cell culture dish containing an appropriate volume of medium.

Seeding PSCs in a 500 mL flask

Day –2: Seed 1.5 x 10^7 PSCs in 100 mL of StemScale medium containing 10 µM ROCK inhibitor (Y-27632). Culture for 24 hr.

Day –1: Perform a 50% medium change with StemScale medium by tipping the flask at a 45° angle and allowing spheroids to settle for 4 min before removing and replacing 50 mL of the medium.

Neural induction in a 500 mL flask

1. After passaging and maintaining PSC spheroids in StemScale medium for 2 days, the average spheroid diameter at the time of induction should be between ~200 µm and 280 µm.

   Note: Spheroid size at the start of differentiation and/or the length of time maintained as spheroids (2 days or 3 days) may need to be optimized depending on cell line.

2. Induction D0: Perform an 80% medium change, replacing 80 mL of StemScale medium with PSC Neural Induction Medium. Return cells to the orbital shaker at 80 rpm. Incubate cells for 48 hr.

3. Induction D2: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 80 rpm. Incubate cells for 24 hr.

4. Induction D3: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 80 rpm. Incubate cells for 24 hr.

5. Induction D4: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 80 rpm. Incubate cells for 24 hr.

6. Induction D5: Perform an 80% medium change with PSC Neural Induction Medium. Return cells to the orbital shaker at 80 rpm. Incubate cells for 24 hr (see Note below).

7. Induction D6: At day 6 of induction, cells should be >90% SOX1/nestin co-positive and are ready to go into Neural Expansion medium.

   Note: Due to proliferation of cells during induction, aggregates may need to be divided into multiple flasks between days 6 and 8 of the protocol. Monitor the medium daily. If the medium is yellow 24 hr after a medium change, divide the aggregates from a single flask into multiple flasks (aggregate dissociation is not recommended at this stage). A 1:2 or 1:3 flask distribution is recommended. The distribution ratio should not be too high, as the agitation dynamics can change and damage the aggregates if too few are in each flask. See the appendix for guidance on well distribution of NSC aggregates.

Neural expansion in 500 mL flasks

After 6 days of induction, NSC aggregates can be evenly distributed into three 500 mL flasks and are ready for neural expansion.

8. Expansion D0: Perform an 80% medium change with Neural Expansion Medium. Return cells to the orbital shaker at 80 rpm for 24 hr. If aggregates have not yet been distributed to multiple flasks, then follow the NSC aggregate well distribution guidance in the appendix, using Neural Expansion Medium.

   Recommended: Neural induction efficiency can be assayed anytime during expansion. Aggregates can be dissociated with StemPro Accutase Cell Dissociation Reagent and plated in 2D on plates coated with Geltrex matrix for ICC analysis of NSC markers SOX1/nestin, and positional markers PAX6/Otx2. See the appendix for guidance on dissociating NSC aggregates and 2D plating for induction efficiency assays, and Figure S2 for expected results.

9. Expansion D1 toD8: Perform an 80% medium change with Neural Expansion Medium. Return cells to the orbital shaker at 80 rpm (Figure 4). Perform 80% medium change daily between D2 and D8. Flasks containing aggregates will likely need to be divided 1:2 between expansion D2 and D4 and again between D6 and D8. Monitor medium consumption daily.

10. Expansion D8: At day 8 of expansion, aggregates can be dissociated and plated in 2D for neuronal maturation, cryopreserved in Neural Expansion Medium with 10% DMSO, or continue to be expanded. See the neuronal maturation section and the appendix for guidance on dissociating and plating NSCs for 2D neuronal maturation. Aggregates can be expanded for at least 10 days without a significant drop in NSC marker expression or impact on subsequent neuronal maturation.

    Note: Between D6 and D8 of expansion, NSC aggregates may need to be divided 1:2 a third time. Monitor medium consumption.

11. Expansion D10 to D12: We recommend plating cells for maturation in 2D culture and/or cryopreserving cells.

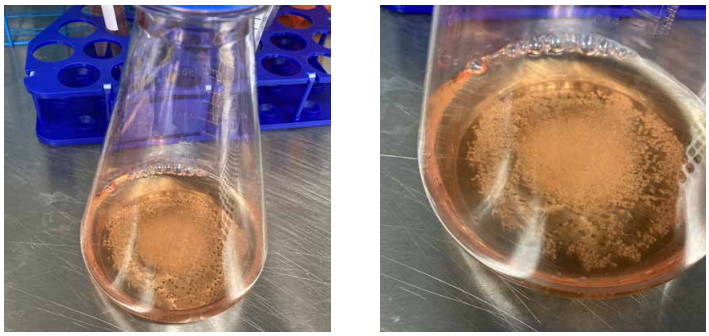

Figure 4. Neural expansion of aggregates. NSC aggregates at day 1 of expansion in a 500 mL flask.

Neuronal maturation in 2D monolayer culture

For neuronal maturation, we recommend plating NSCs in Neuronal Differentiation Medium for 5 days. After 5 days of differentiation, continue with 50% medium changes using Neuronal Maturation Medium (if preferred, maturation can continue in Neuronal Differentiation Medium). Plates should be sequentially coated with poly-D-lysine and then laminin. See the appendix for guidance on media preparation and plate coating.

Aggregate dissociation for neuronal maturation

1. Using a 5 mL serological pipette, gently transfer the medium and expanded NSC aggregates from one well of a 6-well plate to a 15 mL conical tube.
2. Using a 5 mL serological pipette, add 2 mL of DPBS to the well. Tip the plate at a 45° angle. Using the 2 mL of DPBS, rinse any aggregates from the surface of the well. Allow aggregates to settle in the DPBS for ~1 min. Transfer the remaining aggregates and DPBS to the 15 mL conical tube and allow them to settle for ~1 min.
3. Carefully aspirate the medium, leaving ~100–200 µL above the collected aggregates. Gently add 4 mL of DPBS, and swirl the tube to resuspend the aggregates. Allow aggregates to settle for ~1 min.
4. Repeat previous step. Wash steps are necessary for efficient dissociation using StemPro Accutase reagent.
5. Add 2–4 mL of prewarmed StemPro Accutase reagent to the aggregates. The volume of StemPro Accutase reagent added depends on the mass of the collected aggregates. Gently swirl the tube to resuspend the aggregates, and place the tube in a 37°C water bath for 6 min. To facilitate dissociation with StemPro Accutase reagent during incubation, swirl the tube every 1 min to resuspend aggregates.
6. After 5–6 min, gently pipet the aggregates up and down 10 times, using a 1 mL standard-bore pipette tip. Aggregates should begin to dissociate during pipetting. If cell aggregates are still visible, return the tube to the 37°C water bath for 2 min and repeat pipetting using a 1 mL pipette tip. Aggregates should be completely dissociated.
7. Add 6–8 mL of DPBS to the tube to stop the StemPro Accutase dissociation enzyme's activity. Remove a small volume for cell counting. Centrifuge the cell suspension at 300 × *g* for 3 min.
8. Aspirate the medium, leaving ~100 µL of medium above the pelleted cells. Tap the tube several times to dislodge the pellet.

Plating cells for monolayer neuronal maturation

1. Resuspend dissociated NSCs with prewarmed Neuronal Differentiation Medium with 10 µM ROCK inhibitor (Y-27632) to an appropriate density. NSCs can be plated at a seeding density in the range of 2–15 x 10^4 cells/cm^2. Recommended seeding density is 3–8 x 10^4 cells/cm^2. Incubate cells for 24 hr.
2. Perform a 50% medium change with Neuronal Differentiation Medium without ROCK inhibitor.
3. At day 5, perform a 50% medium change with Neuronal Maturation Medium.

   Note: Cells can continue to be matured in Neuronal Differentiation Medium if preferred.
4. Perform a 50% medium change every 2–4 days for the duration of neuronal maturation.

Figure S3 in the appendix shows expected results after 20 days of maturation.

Appendix

Media preparation

PSC Neural Induction Medium:

1. To prepare 500 mL of complete PSC Neural Induction Medium, aseptically mix the following components:

Product	Volume	Cat. No.
Neurobasal Medium	490 mL	Both included in A1647801
Neural Induction Supplement (50X)	10 mL	

2. Complete PSC Neural Induction Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the PSC Neural Induction Medium in a 37°C water bath for no longer than 5–10 min before using. Do not warm the PSC Neural Induction Medium in a 37°C water bath for longer than 10 min, as this may cause degradation of the medium.

Note: Neural Induction Supplement can be thawed at 2–8°C overnight or quickly in a 37°C water bath for about 5 min, and then aliquoted and frozen at –5°C to –20°C to allow for preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing of the Neural Induction Supplement.

Neural Expansion Medium:

1. To prepare 500 mL of complete Neural Expansion Medium, aseptically mix the following components:

Product	Volume	Cat. No.
Neurobasal Medium	245 mL	21103049
Advanced DMEM/F12	245 mL	12634028
Neural Induction supplement (50X)	10 mL	Included in A1647801

2. Complete Neural Expansion Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the Neural Expansion Medium in a 37°C water bath for no longer than 5–10 min before using.

Neuronal Differentiation Medium:

1. To prepare 500 mL of complete Neuronal Differentiation Medium, aseptically mix the following components:

Product	Volume	Cat. No.
Neurobasal Medium	480 mL	21103049
B-27 Supplement (50X)	10 mL	17504044
GlutaMAX Supplement (100X)	5 mL	35050079
CultureOne Supplement (100X)	5 mL	A3320201
Ascorbic acid (1 M)	100 µL	Major laboratory supplier

Neuronal Maturation Medium:

1. To prepare 500 mL of complete Neuronal Maturation Medium, aseptically mix the following components:

Product	Volume	Cat. No.
Neurobasal Plus Medium	480 mL	A3582901
B-27 Plus Supplement (50X)	10 mL	A3582801
GlutaMAX Supplement (100X)	5 mL	35050079
CultureOne Supplement (100X)	5 mL	A3320201

Geltrex matrix coating for NSC induction efficiency assay (ICC)

1. Thaw a vial of Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight. Note: Thawed Geltrex matrix can be aliquoted and then frozen at –5°C to –20°C, or stored at 2–8°C for up to 2 weeks. Avoid repeated thawing and freezing.

2. To create a working solution, dilute the thawed Geltrex matrix solution 1:100 with cold Gibco™ Neurobasal™ Medium or Gibco™ DMEM/F-12 on ice.

3. Quickly cover the whole surface of each culture vessel with the Geltrex matrix solution. Coat with 70 µL per well for a 96-well plate, and with 200 µL per well for a 48-well plate.

4. Incubate the culture vessels in a 37°C, 5% CO_2 incubator for 1 hr.

5. The culture vessels are now ready for use. Just before use, aspirate the diluted Geltrex solution from the culture vessels. Cells can be plated directly onto the Geltrex matrix–coated culture vessels without rinsing. Coated culture vessels can also be stored at 2–8°C for up to one week. When storing, seal the culture vessels with Parafilm™ laboratory film to prevent drying. Before using, warm up the coated culture vessels stored at 2–8°C at room temperature for 30 min.

Sequential coating of culture plates for neuronal maturation

Poly-D-lysine coating:

1. Dilute Gibco™ poly-D-lysine (Cat No. A3890401) in sterile DPBS to prepare a 50 µg/mL working solution.
2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (e.g., 50 µL/well of a 96-well plate).
3. Incubate the vessel at room temperature for 1 hr.
4. Remove the poly-D-lysine solution and rinse the culture vessel surface 3 times with a large volume of distilled water (e.g., 100 µL/well of a 96-well plate). Make sure to rinse the culture vessel thoroughly, as excess poly-D-lysine solution can be toxic to the cells.
5. Remove the final distilled water rinse and leave the coated culture vessel uncovered in the laminar hood to dry. The culture surface will be fully dry after 2 hr. Plates can be used immediately or stored at 4°C.

Laminin coating:

1. Thaw a vial of laminin at room temperature.

   Note: Thawed laminin can be aliquoted and stored at –80°C. Avoid repeated thawing and freezing.
2. Dilute the thawed laminin solution to 5 µg/mL with sterile distilled water to create a working solution.
3. Add the laminin solution into the poly-D-lysine–coated culture vessel to cover the whole surface, and incubate in a 37°C, 5% CO_2 incubator for 1 hr.
4. Immediately prior to seeding cells, aspirate the laminin solution from the coated culture vessel.

NSC aggregate distribution (6-well plate)

1. After performing an 80% medium change, tip the plate with the cells at a 45° angle. With a 5 mL serological pipette, add 1 mL medium for a final volume of 3 mL. Gently pipette the medium up and down one time to get an even suspension of aggregates. Gently remove the entire volume from a single well and divide the aggregates evenly into 3 wells. To collect the remaining aggregates, add 3 mL of medium and distribute the volume evenly into the new wells.
2. Tip up the new plate containing cell aggregates at a 45° angle. Inspect wells to ensure each of the new wells received an approximately even number of medium (2 mL) and total aggregate mass. If well contents are uneven, medium and/or aggregates can be adjusted using a 5 mL serological pipette or a wide-bore 200 µL pipette tip.

NSC aggregate distribution (500 mL flask)

1. Add 50 mL Neural Expansion Medium to a new 500 mL flask.
2. Tip the 500 mL flask containing NSC aggregates at a 45° angle for <1 min, until aggregates have settled.
3. With a 10 mL serological pipette, transfer half of the mass of the aggregates to the new 500 mL flask while keeping track of the volume of medium added. When the two flasks have approximately equal amounts of NSC aggregate mass, add appropriate volumes of Neural Expansion Medium so that each flask contains 100 mL.

NSC aggregate dissociation and 2D plating guidance for induction efficiency assay

1. Using a 48- or 96-well plate, coat a sufficient number of wells with Geltrex matrix at 1:100 dilution in DMEM/F-12, following the guidance provided previously. A 48-well plate can be coated with 200 µL per well of diluted Geltrex matrix. A 96-well plate can be coated with 70 µL per well of diluted Geltrex matrix.
2. Using a 5 mL serological pipette or a wide-bore pipette tip, remove a sample of NSC aggregates from a well and place the sample in a 15 mL conical tube. The number of NSC aggregates needed depends significantly on their size. Aim to remove enough mass to yield approximately 2–4 million cells (about 2–5 aggregates). Replace the volume of expansion medium removed from the well.

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3. Let the aggregates settle to the bottom of the tube and estimate the appropriate mass to equal 2–4 million cells. Add or remove aggregates as necessary. Aspirate the existing medium from the 15 mL conical tube, leaving ~100 µL so as to not remove any of the collected cell aggregates. Gently add 3 mL of DPBS without calcium and magnesium. Let the cell aggregates settle to the bottom of the tube and repeat. Aspirate the DPBS solution, leaving approximately 100 µL.

4. Add 2 mL of prewarmed StemPro Accutase reagent to the cell aggregates. Gently swirl the tube to resuspend the cell aggregates, and place the tube in a 37°C water bath for 5–6 min. During incubation with StemPro Accutase reagent, swirl the tube every 1–2 min. After 5–6 min, gently pipet the cell aggregates up and down 10 times using a standard-bore 1 mL pipette. Cell aggregates should begin to dissociate during pipetting. If cell aggregates are still visible, return the tube to the incubator for 2 min and repeat pipetting with a 1 mL pipette. Cells should be completely dissociated.

5. Add 4 mL of DPBS without calcium and magnesium. Mix the cell suspension and remove a volume of cells for counting. Centrifuge the cell suspension at 300 x *g* for 3 min. Count cells.

6. After centrifugation aspirate the supernatant, leaving the pelleted cells and ~100 µL of medium. Gently tap the tube to dislodge the cells. Resuspend the cells in an appropriate volume of Neural Expansion Medium containing 10 µM ROCK inhibitor (Y-27632). The cell suspension should have 10^6 cells/mL. Plate cells at 2.5 x 10^5 cells/cm^2 in 100 µL per well for a 96-well plate, or 250 µL per well for a 48-well plate.

7. Incubate cells stationary in a cell culture incubator. After 24 hr, change the medium with Neural Expansion Medium without ROCK inhibitor. After 48 hr, cells can be fixed with 4% paraformaldehyde and stained with appropriate antibodies.

Supplemental figures

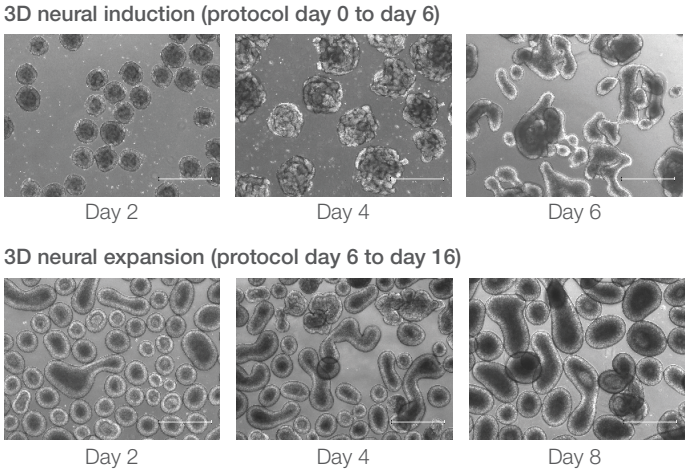

Figure S1. Cell aggregate morphology during induction and expansion. Changes in aggregate morphology over 6 days of induction or 8 days of expansion. Aggregates initially expand in size before forming smaller aggregates with neuroepithelial-like characteristics.

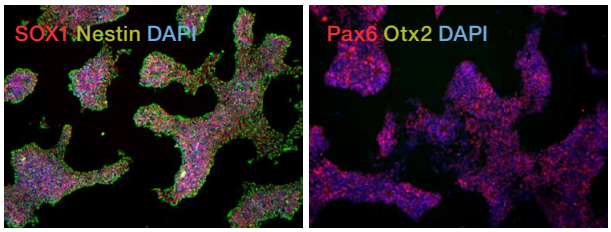

Figure S2. NSC marker expression after 6 days of induction. Neural induction in suspension culture from PSC spheroids grown in StemScale medium yield highly pure >90% SOX1/nestin co-positive cells after 6 days of induction. Positional marker staining showed ~40% Pax6-positive cells and <5% Otx2-positive cells. Equivalent results are obtained from the monolayer neural induction method.

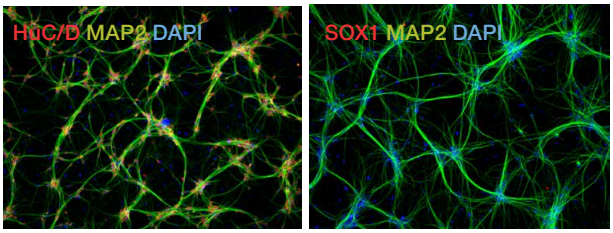

Figure S3. Maturation markers after 20 days of maturation. After 20 days of neuronal maturation of NSCs, the culture population is enriched with mature neurons (HuC/D and MAP2) with a few expanded progenitors (SOX1).

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