

Generating mature dopaminergic neurons from pluripotent stem cell spheroids

Introduction

This protocol is for researchers who culture pluripotent stem cells (PSCs) in Gibco™ StemScale™ PSC Suspension Medium and want to generate dopaminergic (DA) neurons for downstream high-throughput applications. Figure 1 describes the workflow from PSC spheroids to mature DA neurons using the Gibco™ PSC Dopaminergic Neuron Differentiation Kit. During differentiation, PSC spheroids are first induced into midbrain-specified floor plate (FP) progenitor cell aggregates using FP Specification Medium. Next, FP progenitor cell aggregates are expanded in FP Cell Expansion Medium. Finally, the aggregates are dissociated and differentiated in monolayer culture to mature DA neurons in DA Neuron Maturation Medium. A protocol is provided for performing suspension culture DA neuron differentiation in a 6-well plate format as well as guidance for large-scale culture in 500 mL flasks and optimization. The 6-well plate format is beneficial for initial optimization of suspension culture differentiation before scaling up to large-scale 500 mL flasks.

PSCs for this procedure should be adapted to StemScale medium (at least 3 passages) and can be cultured as 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™ CO₂ Resistant Shaker (Cat. No. 88881101).

Different shaker platforms may need additional optimization of agitation speed settings. See the appendix for media preparations, additional guidance, and supplemental figures.

FP specification and expansion (6-well plate)

The first step of differentiation is specification of PSC spheroids grown in StemScale medium to midbrain-specified FP progenitor cell aggregates in FP Specification Medium.

FP expansion in 6-well plate

1. FP specification, day –2: Passage PSCs from either 2D culture or StemScale medium. Dissociate cells with Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent to obtain a single-cell suspension. Plate 3×10^5 viable cells per well in 2 mL of StemScale medium containing 10 μ M ROCK inhibitor (Y-27632) in non-tissue culture treated 6-well plates. Maintain cells on an orbital shaker platform at 70 rpm in a 37°C incubator. Incubate cells for 24 hours.
2. FP specification, day –1: Perform a 50% medium replacement with 1.0 mL StemScale medium. Tilt plates at a ~45° angle for ~3 min to ensure spheroids settle via gravity sedimentation. With the plate still tilted, carefully remove 50% of the medium (1 mL) from each well. (Note: Always aspirate with the pipette tip in the center of the well, as far from the spheroid pellet as possible.) Add an equal volume (1 mL) of StemScale medium to replace the volume that has been removed. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 24 hours.

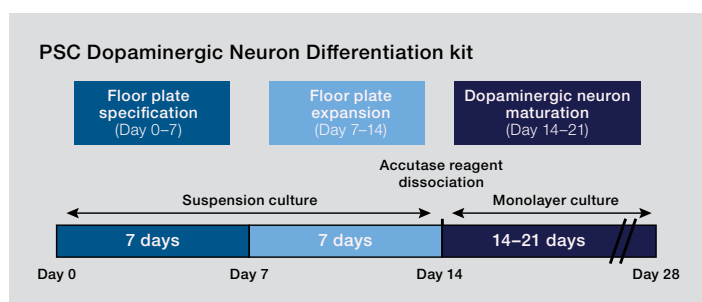


Figure 1. Workflow for generation of DA neurons. For this protocol, FP specification and expansion are performed in suspension culture, whereas maturation is performed in monolayer culture.

- FP specification, day 0: The size of the PSC spheroids at day 0 is a critical parameter for optimal specification efficiency. The average spheroid diameter to start FP specification should be ~210–260 μm (Figure 2). When spheroids are at an appropriate size, perform an 80% medium change with FP Specification Medium. Repeat the 80% medium change with FP Specification Medium. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 48 hours.

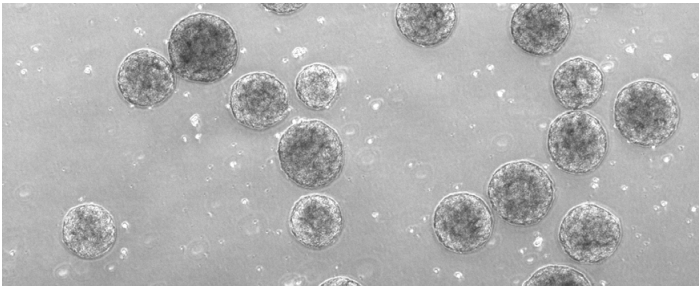


Figure 2. PSC spheroids at day 0.

- FP specification, day 2: At day 2 of specification, the cell aggregates will have a distinct epithelial-like morphology (Figure 3). To decrease stress on the cell aggregates, increase the volume of medium in each well during the medium change. Using a 5 mL pipette, change the medium by removing 1.5 mL and replacing it with 2.0 mL of FP Specification Medium. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 48 hours.

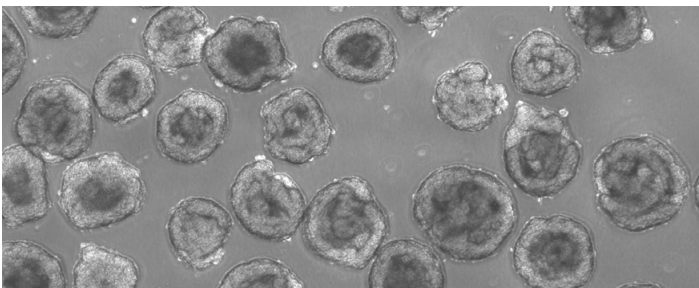


Figure 3. Cell aggregates at day 2 of specification.

- FP specification, day 4: Perform an 80% medium change with FP Specification Medium. Return plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 24 hours.
- FP specification, day 5: Perform an 80% medium change with FP Specification Medium. Return plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 24 hours.
- FP specification, day 6: Using a 5 mL pipette, perform medium change by removing 1.5 mL and replacing with 2.0 mL of FP Specification Medium. Return plate

to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate cells for 24 hours.

- FP specification, day 7: Cells should be appropriately specified and ready for the expansion step. See Figure 5 in the appendix for expected aggregate morphology during FP specification.

FP expansion in 6-well plate

The second step of differentiation is the expansion of FP progenitor cell aggregates in FP Cell Expansion Medium for at least 7 days. Expansion increases the cell number and improves the efficiency of differentiation towards DA neurons. FP progenitor cell aggregates have been maintained in expansion medium for at least 10 days without loss of FP progenitor cell markers and with no impact on subsequent neuronal maturation efficiency.

- FP expansion, day 0: Perform an 80% medium change with FP Cell Expansion Medium. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 24 hours.
- FP expansion, day 2: As FP progenitor cell aggregates expand, they will require frequent medium changes. To avoid daily medium changes, aggregates from each well should be divided into three wells (Figure 4). To divide spheroids into multiple wells, obtain an appropriate number of non-tissue culture treated 6-well plates (for example, one 6-well plate will require three 6-well plates). Add 2.5 mL of FP Cell Expansion Medium to each well of the new plate. Tilt the plate containing aggregates at a 45° angle for until aggregates have settled to the bottom. Using a 5 mL serological pipette, carefully collect the aggregates and divide them roughly equally into three wells. When finished, return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate cells for 48 hours.

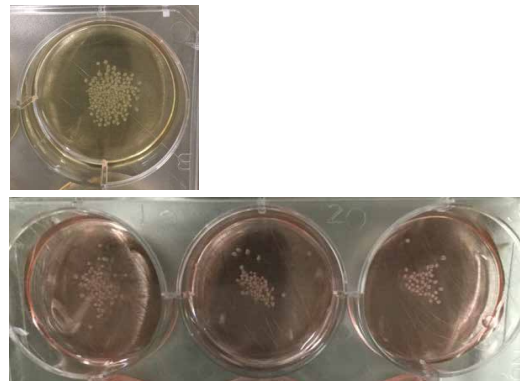


Figure 4. Division of aggregates from one well (top) into 3 wells (bottom).

3. FP expansion, day 4: Perform an 80% medium change with FP Cell Expansion Medium. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 48 hours.
4. FP expansion, day 6: Perform an 80% medium change with FP Cell Expansion Medium. Return the plate to the orbital shaker platform at 70 rpm in the 37°C incubator. Incubate the cells for 24 hours.
5. FP expansion, day 7: After 7 days of expansion, cell aggregates can be dissociated and plated for 2D neuronal maturation or cryopreserved.

After the FP specification step, >80% of cells should show co-expression of the FP markers FOXA2 and OTX2. FP specification efficiency can be assayed any time after day 7 of FP specification. Guidance for the FP specification efficiency assay can be found in the next section. See the appendix for expected results.

Guidance for FP specification and expansion, 500 mL flask format

To scale up from the 6-well plate format to a flask format, some optimization may be required. The goal is to achieve similar hydrodynamic conditions present in the 6-well plate. Important variables to consider for scale-up optimization in flask format are the volume of the medium and shaker platform speed.

For 500 mL flasks, we have found that 100 mL of medium and a shaker platform speed of 70 rpm produces similar results as the 6-well plate format. At day -2, PSCs should be seeded at the same concentration of StemScale medium as in the 6-well plate, 1.5×10^5 cells/mL.

It is important to monitor PSC spheroid size before starting FP specification. To observe PSC spheroids or cell aggregates under a microscope throughout differentiation, transfer a sample amount from the flask to a cell culture vessel with an appropriate volume of medium.

The same protocol timeline for FP specification and expansion can be followed, as well as the medium change frequency. The volume of medium in the flask should remain at 100 mL throughout.

Due to proliferation of cells during expansion, we recommend FP cell aggregates be divided into multiple flasks (FP aggregates from one flask should be divided into two flasks) between day 8 and day 12 of protocol. Monitor the medium daily.

FP specification efficiency assay

1. Coat a sufficient number of wells of a 48-well plate with Gibco™ Laminin (Cat. No. 23017015) at a 1:100 dilution (10 µg/mL) in sterile distilled water at room temperature.
2. Using a 5 mL serological pipette or a pipette with a wide-bore 200 µL tip, remove a sample of FP progenitor cell aggregates from a well and place the sample in a 15 mL conical tube. The number of aggregates depends significantly on the size, so aim to remove enough cell mass to yield approximately $1-2 \times 10^6$ cells. Depending on the size, 3 to 4 aggregates should yield $1-2 \times 10^6$ cells. Replace the volume of expansion medium removed from the well.
3. Let the aggregates settle to the bottom of the tube and estimate the appropriate cell mass for $2-3 \times 10^6$ cells. Add or remove aggregates as needed. Aspirate the existing medium from the 15 mL conical tube, leaving ~100 µL as to not aspirate any of the collected aggregates. Gently add 3 mL of DPBS (no calcium, no magnesium). Let the spheres settle to the bottom of the tube and repeat the DPBS wash. Aspirate the DPBS solution, leaving approximately 100 µL.
4. Add 2 mL of pre-warmed StemPro Accutase reagent to the aggregates. Gently swirl the tube to resuspend the aggregates, and place the tube in a 37°C water bath for 5–6 min. To facilitate dissociation during incubation with StemPro Accutase reagent, swirl the tube every 1–2 min to resuspend aggregates. After 5–6 min, gently pipet the aggregates up and down 10 times, using a pipette with a standard-bore 1,000 µL tip. Aggregates should begin to dissociate during pipetting.
5. If cell aggregates are still visible, return the tube to the 37°C water bath for 2–3 min. Gently pipet the cell suspension up and down using a pipette with a standard-bore 1,000 µL tip until all of the spheres are dispersed into a single-cell suspension. Pass the cell suspension through a 100 µm strainer, and then rinse the mesh with 4 mL of DPBS.
6. Add 4 mL of DPBS. Remove a volume of cells for counting, and spin the cells down at $300 \times g$ for 3 min. Count the cells.

- 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 FP Cell Expansion Medium containing 10 μM ROCK inhibitor (Y-27632). The cell suspension should have 10^6 cells/mL. Plate the cells at 2.0×10^5 cells/cm², or 200 μL /well.
- Incubate the cells stationary in a cell culture incubator. 24 hours after plating, add 200 μL FP Cell Expansion Medium without ROCK inhibitor. 48 hours after plating, the cells can be fixed with 4% paraformaldehyde, blocked, and stained with the FP-specific antibodies FOXA2 and OTX2.
- After 5–6 min, gently pipet the aggregates up and down 10 times, using a 1,000 μL standard-bore tip. Aggregates should begin to dissociate during pipetting. If cell aggregates are still visible, return the tube to the 37°C water bath for 2–5 min and repeat pipetting with a 1,000 μL standard-bore tip. Aggregates should be completely dissociated.*
- Add 4–6 mL of DPBS to stop the StemPro Accutase dissociation enzyme. Remove a small volume for cell counting. Centrifuge the cell suspension at 300 x g for 3 min.

DA neuron maturation in monolayer 2D culture

The last step of DA neuron differentiation is the maturation of FP progenitor cells into mature midbrain DA neurons. FP progenitor cells are matured in DA Neuron Maturation Medium on culture vessels sequentially coated with Gibco™ poly-D-lysine and laminin (see the appendix for coating guidance). The recommended seeding density is 2.0×10^5 cells/cm². The seeding density range is $1.5\text{--}2.5 \times 10^5$ cells/cm². Higher maturation seeding densities increase the percentage of TH-positive DA neurons.

- Using a 5 mL serological pipette, gently transfer the medium and expanded FP progenitor aggregates from one well of a 6-well plate to a 15 mL conical tube.
- Using a 5 mL serological pipette, add 1 mL of DPBS (no calcium, no magnesium) to the well. Swirl the plate and tip the plate at a 45° angle, allowing the remaining aggregates to settle to the bottom of the well. Transfer the remaining aggregates to the 15 mL conical tube and allow them to settle for ~30 sec.
- Aspirate the existing medium, leaving ~100–200 μL above the collected aggregates. Gently add 3 mL of DPBS and swirl the tube to resuspend the aggregates. Allow the aggregates to settle for ~30 sec.
- Repeat the previous step two times.
- Add 2 mL of pre-warmed StemPro Accutase reagent to the 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 during incubation with StemPro Accutase reagent, swirl the tube every 1–2 min to resuspend aggregates.
- Resuspend the cell pellet in DA Neuron Maturation Medium with 10 μM ROCK inhibitor (Y-27632).
- Seed the sequentially coated culture plates with the dissociated cells at a seeding density of $1.75\text{--}2.5 \times 10^5$ cells/cm² in DA Neuron Maturation Medium with 10 μM ROCK inhibitor (Y-27632). Lower seeding densities are not recommended.
- Incubate the cells without shaking overnight at 37°C in a humidified atmosphere of 5% CO₂.
- At day 1 of maturation, perform a 50% medium change with DA Neuron Maturation Medium without ROCK inhibitor (Y-27632).
- For subsequent feeds (every 2–3 days), aspirate half of the spent medium and replace it with DA Neuron Maturation Medium.
- Mature neurons can be visualized as early as 8 days after the addition of DA Neuron Maturation Medium, but for optimal results we recommend culturing cells in maturation medium for at least 14 days. The expected yield of TH-positive neurons is ~30–50%.
- For long-term culture of DA neurons >14 days, we recommend 50% medium changes with Gibco™ Neurobasal™ Plus basal medium (standard Neurobasal medium with 0.5 mM Gibco™ GlutaMAX™ Supplement can also be used) supplemented with DA Neuron Maturation Supplement (1X final concentration). DA neuron cultures can be maintained for over 28 days.

* Variability in duration of dissociation of FP progenitor aggregates with StemPro Accutase reagent has been observed. Typically, the aggregates dissociate within 6–12 min with the described pipetting steps; however, longer treatment with StemPro Accutase reagent has been necessary on some occasions. Up to 30 min treatment has been performed without significant impact on viability. The presence of small aggregates of undissociated cells is not detrimental to the maturation step, as the cells will plate down and spread out on the sequentially coated plates. If the remaining aggregates are not wanted, pass the cell suspension through a 100 μm strainer, and then rinse the mesh with 4–6 mL of DPBS.

Appendix

Sequential coating of culture plates

Poly-D-lysine coating

1. Dilute poly-D-lysine (Cat. No. A3890401) solution in sterile DPBS (no calcium, no magnesium) 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 hour.
4. Remove the poly-D-lysine solution and rinse the culture 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 flow hood to dry. The culture surface will be fully dry after 2 hours. Plates can be used immediately or stored at 4°C.

Laminin coating

1. Thaw a vial of laminin (Cat. No. 23017015) 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% CO₂ incubator for 1 hour.
4. Immediately prior to seeding cells, aspirate the laminin solution from the coated culture vessel.

Media preparation

FP Specification Medium

Product	Volume	Cat. No.
Neurobasal Medium	95 mL	21103049
FP Specification Supplement (20X)	5 mL	A3146801

1. Thaw the FP Specification Supplement (20X) at 4°C overnight, or at room temperature (15–25°C) for 30 min.
2. Add 5 mL of 20X FP Specification Supplement to 95 mL of Neurobasal Medium, and mix well.
3. Store the complete FP Specification Medium at 4°C and use within 2 weeks. On the day of use, take the volume that is needed for that day and warm it at 37°C; avoid repeated warming.

FP Cell Expansion Medium

Product	Volume	Cat. No.
FP Cell Expansion Base Medium	490 mL	A3165801
FP Cell Expansion Supplement (50X)	10 mL	A3165801

1. Store FP Cell Expansion Base Medium at 4°C and FP Cell Expansion Supplement (50X) at –20°C until use. Do not prepare complete expansion medium until needed.
2. Thaw the FP Cell Expansion Supplement (50X) at 4°C overnight, or at room temperature (15–25°C) for 1 hour.
3. Remove 10 mL of medium from the bottle of FP Cell Expansion Base Medium and discard.
4. Add 10 mL of FP Cell Expansion Supplement (50X) to the remaining 490 mL of FP Cell Expansion Base Medium and mix well.
5. Store the complete FP Cell Expansion Medium at 4°C and use within 2 weeks. On the day of use, take the volume that is needed for that day and warm it at 37°C; avoid repeated warming.

DA Neuron Maturation Medium

Product	Volume	Cat. No.
DA Neuron Maturation Supplement (50X)	10 mL	A3147401
DMEM/F-12	490 mL	10565042

1. Store the DA Neuron Maturation Supplement (50X) at -20°C until use. Do not prepare complete maturation medium until needed.
2. Thaw the DA Neuron Maturation Supplement (50X) at 4°C overnight, or at room temperature ($15\text{--}25^{\circ}\text{C}$) for 1 hour.

3. Add 10 mL of DA Neuron Maturation Supplement (50X) to 490 mL of DMEM/F-12 medium, and mix well.
4. Store the complete DA Neuron Maturation Medium at 4°C and use within 2 weeks. On the day of use, take the volume that is needed for that day and warm it at 37°C ; avoid repeated warming.

Expected results

Representative results for morphology of aggregates, staining of FP markers, and DA neuron maturation are shown in Figures 5–7.

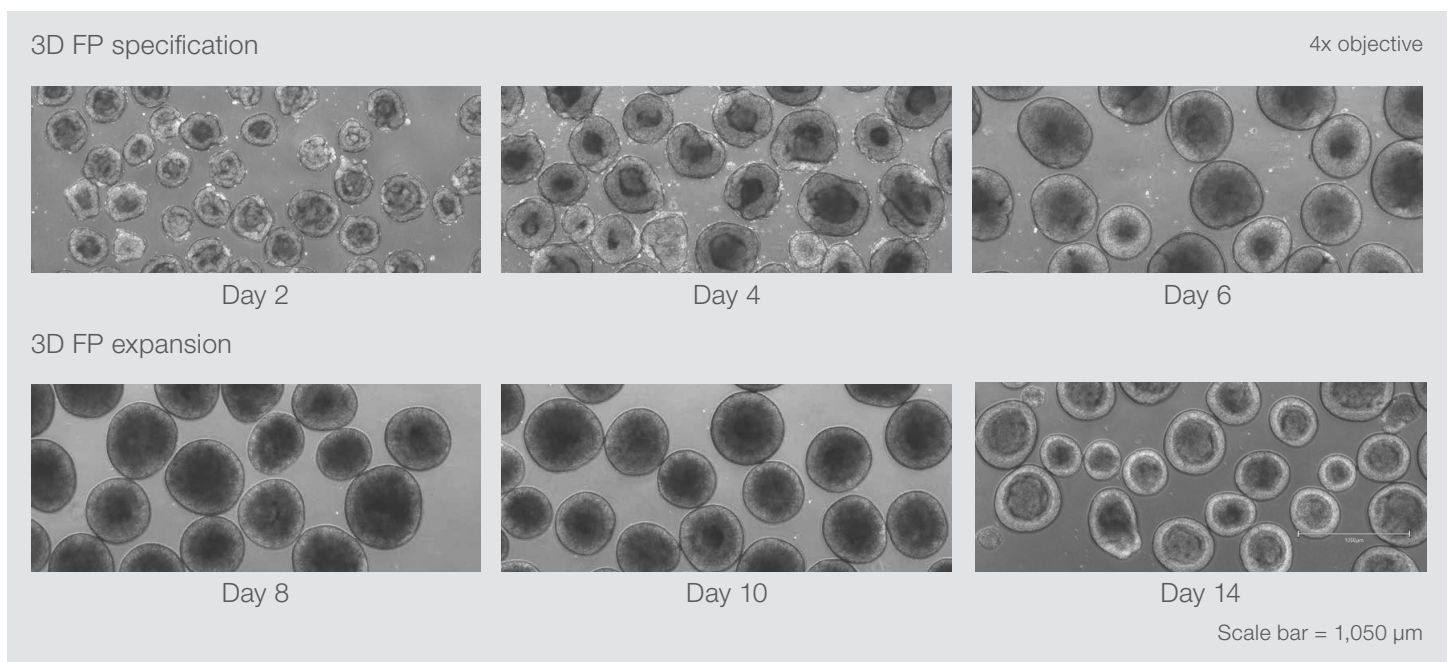


Figure 5. Expected morphology of aggregates during FP specification and expansion.

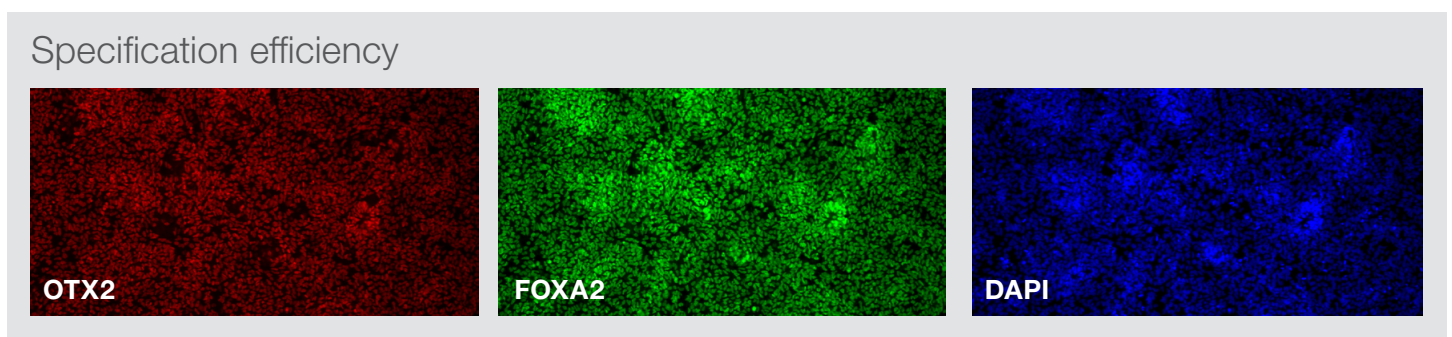
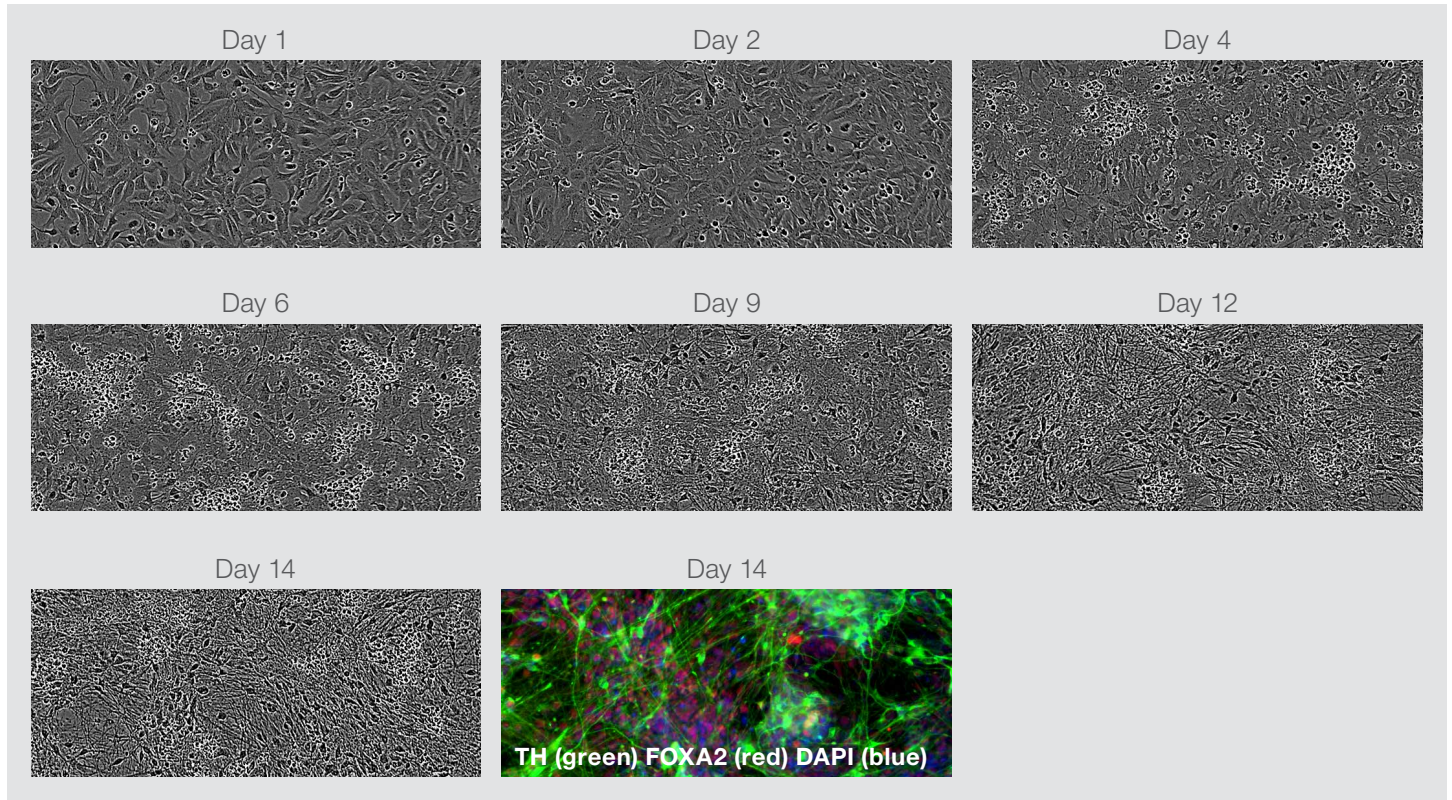


Figure 6. Expected results for FP specification. Cells were stained using antibodies specific to OTX2 and FOXA2. Nuclei were stained with DAPI.

A



B

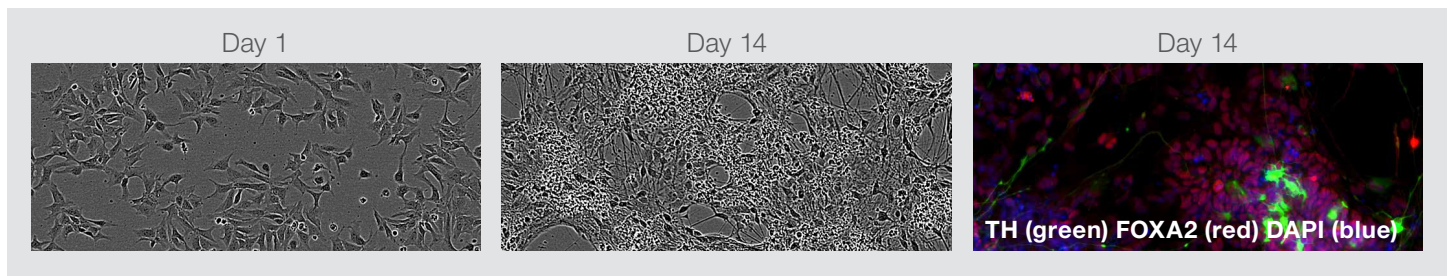


Figure 7. Expected results for DA neuron maturation. (A) The recommended seeding density of 2.0×10^5 cells/cm² was used. **(B)** Impact of low seeding density (0.75×10^5 cells/cm²) on TH-positive neurons.

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