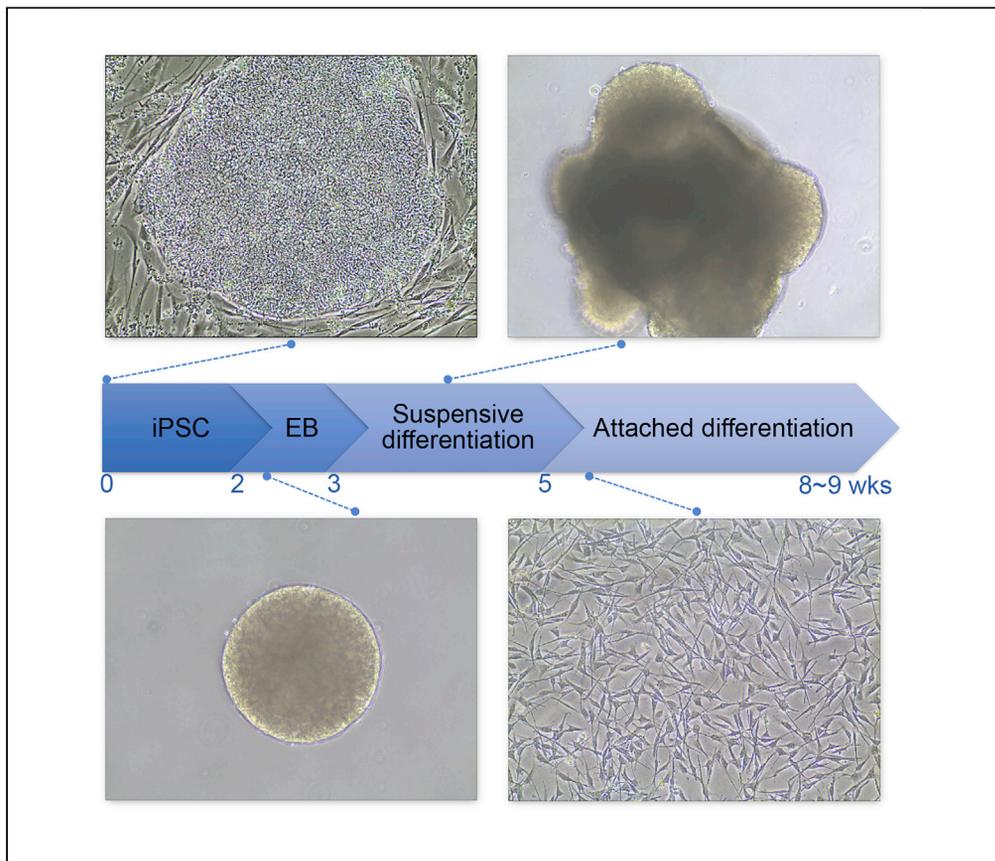


Protocol

Generation of Human iMelanocytes from Induced Pluripotent Stem Cells through a Suspension Culture System



Melanocytes, derived from neural crest cells, are involved in melanin production. This protocol describes a method to generate induced melanocytes (iMelanocytes) from human induced pluripotent stem cells (iPSCs) using a suspension culture system, which considerably improves the differentiation efficiency. The most critical parts of this protocol are the selection of a reliable iPSC line with strong potential to differentiate into melanocytes and their stemness maintenance.

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HIGHLIGHTS

Selection of reliable
iPSC lines and their
stemness
maintenance is
critical

EB-based suspensive
system enhances
efficiency of
iMelanocytes
generation

EBs characterized by
smooth borders and
dark centers without
cavities are preferred

Quality control is
performed through
morphology checks
and melanocyte
markers

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Protocol

Generation of Human iMelanocytes from Induced Pluripotent Stem Cells through a Suspension Culture System

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SUMMARY

Melanocytes, derived from neural crest cells, are involved in melanin production. This protocol describes a method to generate induced melanocytes (iMelanocytes) from human induced pluripotent stem cells (iPSCs) using a suspension culture system, which considerably improves the differentiation efficiency. The most critical parts of this protocol are the selection of a reliable iPSC line with strong potential to differentiate into melanocytes and their stemness maintenance.

For complete information on the use and generation of this protocol, please refer to our *Cell Reports* article, Liu et al. (2019).

BEFORE YOU BEGIN

Human Embryonic Stem (hES) Cell Medium

⌚ TIMING: ~20 min

1. Combine the following reagents, filter-sterilize, and aliquot 45 mL/tube.

400 mL DMEM/F-12 Medium

100 mL Knockout serum replacement

5 mL 100 mM GlutaMAX

5 mL Non-Essential Amino Acids

1 μL 55 M 2-Mercaptoethanol

2. Store at 4°C for up to 14 days, or –20°C for up to one year.



- When you are ready to use the medium, add 45 μL of 4 $\mu\text{g}/\text{mL}$ bFGF solution to each tube and use the medium within 14 days.

Dissociation Solution for Human iPSC Passaged on MEF

⌚ TIMING: ~20 min

- Combine the following reagents, filter-sterilize, and aliquot 10 mL/tube.
- Store at 4°C for up to 14 days, or –20°C for up to one year.

34.5 mL DPBS

10 mL Knockout serum replacement

5 mL 2.5% trypsin

500 μL 100 mM $\text{CaCl}_2/\text{DPBS}$

L Wnt-3A Conditioned Medium

⌚ TIMING: ~7 days

- Culture L Wnt-3A cells in DMEM (high glucose) supplemented with 10% FBS.
- Change the medium every 2–3 days.
- When the cells reach 70%–80% confluence, remove and discard the culture medium.
- Wash the cells with DPBS.
- For 100 mm dish, add 2 mL 0.05% Trypsin-EDTA solution and observe the cells under an inverted microscope.
- When the cells become round, add 10 mL DMEM supplemented with 10% FBS and pipette cells gently.
- Collect the cell suspension and centrifuge at 300 $\times g$ for 5 min at 4°C. Discard the supernatant.
- Resuspend the pellet in DMEM supplemented with 1% FBS.
- Split the cells 1:10 in 100 mm dishes with 10 mL medium for each dish.
- After 4 days of growth, collect the medium and filter-sterilize (first batch of conditioned medium). Store at 4°C.
- Add 10 mL fresh medium (DMEM supplemented with 1% FBS) and culture for 3 days.
- Collect the medium and filter-sterilize (second batch of conditioned medium).
- Mix the first and second batch of medium 1:1 to form L Wnt-3A conditioned medium.
- Aliquot 25 mL/tube and store at –80°C.

Note: To collect the conditioned medium, it is recommended to use cells that have been passaged at least twice after thawing to ensure the cells have returned to normal status.

Note: After collection of the conditioned medium, discard the cells because they have been cultured in abnormal medium and they are also over-confluent.

Melanocyte Differentiation Medium (50 mL)

⌚ TIMING: ~30 min

- Mix the following components in Part I, filter-sterilize, and then mix with the components in Part II.
- Store at 4°C for up to 14 days. Protect from light.
 - Part I:

- a. 15 mL DMEM (low-glucose)
 - b. 10 mL MCDB 201 medium
 - c. 1 μ L 2.5 mM Dexamethasone
 - d. 2.8 μ L 0.03 mg/mL Cholera Toxin
 - e. 2.5 μ L 1 mM Phorbol 12-myristate 13-acetate (TPA)
 - f. 100 μ L 10 mg/mL L-Ascorbic acid
2. Part II:
- a. 25 mL L Wnt-3A conditioned medium
 - b. 250 μ L 10 μ g/mL Stem cell factor (SCF)
 - c. 50 μ L 100 μ M Endothelin-3
 - d. 50 μ L 4 μ g/mL bFGF
 - e. 500 μ L 100x Linoleic acid-albumin from bovine serum albumin
 - f. 500 μ L 100x Insulin-transferrin-selenium
 - g. 500 100x GlutaMAX

Note: We referred to the original protocol for hESCs-derived melanocytes differentiation using Wnt-3A conditioned medium (Fang et al., 2006) and established this conventional and practical system with expected results.

Alternatives: Purified Wnt-3A can be substituted for Wnt-3A conditioned medium; however, this reduced melanocyte differentiation efficiency (Fang et al., 2006). Wnt-3A protein was found to be effective as described by Ohta et al., 2011, though the purified protein was not directly compared to conditioned medium.

Matrigel-Coated Plate

⌚ TIMING: ~60 min

1. For each well of a 6-well plate, add 750 μ L Matrigel (diluted in DMEM/F12 medium, 1:80 v/v) with pre-cooled tips. Ensure that the solution covers the entire bottom of the well.
2. Incubate the plate at 37°C for at least 1 h.
3. Aspirate the Matrigel from the coated plate. Ensure the tip of the pipette does not scratch the coated surface.
4. Wash once with DMEM/F12 medium and discard.
5. Add 1 mL mTeSR1 medium.

Fibronectin-Coated Plate

⌚ TIMING: ~ 60 min

1. For each well of a 6-well plate, add 1 mL DPBS and 20 μ L 1 mg/mL fibronectin solution. Mix sufficiently and cover the entire bottom of the well.
2. Incubate at 15–25°C for at least 1 h.
3. Discard the solution and wash once with 1 mL DPBS, to carefully remove residual fibronectin. Avoid scratching the coated surface.

Selection of iPSC Lines

⚠ **CRITICAL:** Different iPSC lines show different potentials towards melanocyte differentiation (Figure 1). Therefore, it is critical to select or screen proper cell lines for this purpose.

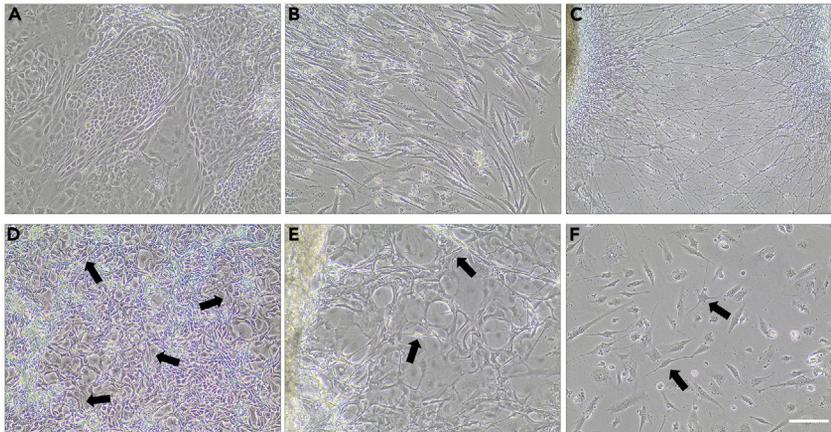


Figure 1. Some iPSC Lines Have No or Low Potential to Differentiate into Melanocytes

Different cellular morphologies (a–c) can be observed during the process of differentiation (week 3, before single cell dissociation), such as typical epithelium-like (a, cobblestone morphology), fibroblast-like (b, bipolar or multipolar with elongated shapes) or neuron-like cells (c, with long thin axon). Although limited melanocyte-like cells could be found sometimes by week three (d, e, arrows), they fail to proliferate after single-cell dissociation (f, arrows). Arrows: melanocyte-like cells. Scale bar: 200 μm .

Note: For researchers who lack experience in this aspect, it is best to start with a standard cell line, such as hES H9, which has a robust ability to differentiate into melanocytes (Fang et al., 2006).

Alternatives: iPSC clone 201B7 (Takahashi et al., 2007, Hosaka et al., 2019) and WTc11 (https://www.coriell.org/0/Sections/Search/Sample_Detail.aspx?Ref=GM25256) can be used as alternatives if hES cells are unavailable.

Optional: Select an iPSC line through evaluation of the formation of EBs (Figures 2C and 2D), or through the detection the expression of specific markers, such as SALL3 at day 7 of EB formation, to predict the potential of differentiation into melanocytes (Guo et al., 2019).

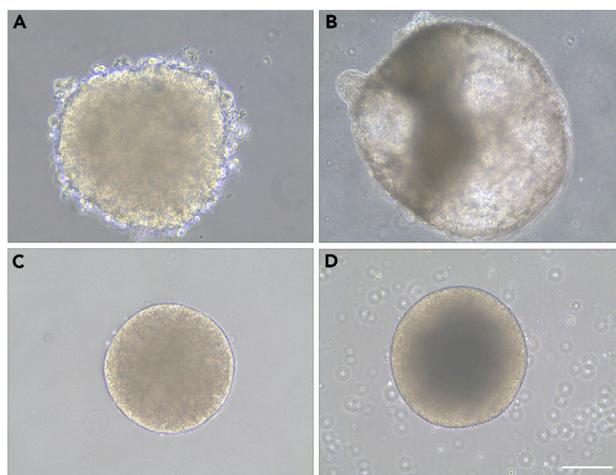


Figure 2. Bad EBs and Good EBs

EBs in a poor state have blurry boundaries (a) or cavities (b), while EBs in a good state always have a smooth border and dark center (c, d). Scale bar: 200 μm .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Monoclonal anti-MITF	Sigma-Aldrich	Cat#M6065; RRID:AB_260607
Mouse Monoclonal anti-SOX10	Abcam	Cat#ab212843
Rabbit Polyclonal anti-PAX3 (hPAX3)	Atlas Antibodie	Cat#HPA063659; RRID:AB_2685080
Mouse Monoclonal anti-TYRP1 (clone TA99)	Millpore	Cat#MABC592
Alexa Fluor 488 Goat anti-Mouse IgG1	Thermo Fisher Scientific	Cat#A21121; RRID:AB_2535764
Cy™3 Goat Anti-Mouse IgG2b	Jackson ImmunoResearch Labs	Cat#115-165-207; RRID:AB_2338696
Cy™3 Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	Cat#111-165-144; RRID:AB_2338006
Alexa Fluor 488 Goat anti-Mouse IgG2a	Thermo Fisher Scientific	Cat#A21131; RRID:AB_2535771
Chemicals, Peptides, and Recombinant Proteins		
DMEM/F12	GIBCO	Cat#11330032
Knockout serum replacement (KSR)	GIBCO	Cat#10828028
GlutaMAX™	GIBCO	Cat#35050061
Non-Essential Amino Acids Solution	GIBCO	Cat#11140050
Basic fibroblast growth factor (bFGF)	Wako	Cat#068-04544
2-Mercaptoethanol	GIBCO	Cat#21985-023
DPBS	HyClone	Cat# SH30256.01B
2.5% trypsin	GIBCO	Cat#15090046
CaCl ₂	Sigma-Aldrich	Cat#V900266
DMEM high glucose	Sigma-Aldrich	Cat#D5796
Fetal bovine serum (FBS)	GIBCO	Cat#A3260802
0.05% Trypsin-EDTA	GIBCO	Cat#25300120
DMEM low glucose	GIBCO	Cat#10567014
MCDB 201 Medium	Sigma-Aldrich	Cat#M6770
Dexamethasone	Sigma-Aldrich	Cat#D4902
Cholera toxin	Sigma-Aldrich	Cat#C8052
12-O-tetradecanoyl-phorbol 13-acetate (TPA)	Sigma-Aldrich	Cat#P8139
L-ascorbic acid	Sigma-Aldrich	Cat#A4403
Stem cell factor (SCF)	R & D	Cat#255-SC-050
Endothelin-3	ENZO	Cat#ALX-155-003-PC05
Insulin-transferrin-selenium	Sigma-Aldrich	Cat#I3146
Linoleic acid-bovine serum albumin	Sigma-Aldrich	Cat#L9530
Fibronectin	BD Biosciences	Cat#356008
Gelatin	Sigma-Aldrich	Cat#G9391
Y-27632	Wako	Cat#253-00513
Matrigel	Corning	Cat#354277

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
mTeSR™1	StemCell Technologies, Inc.	Cat#85850
ReLeSR™	StemCell Technologies, Inc.	Cat#05872
ACCUTASE™	Innovative Cell Technologies	Cat#AT104-500
AggreWell™ EB Formation Medium	StemCell Technologies, Inc.	Cat#05893
TrypLE™ Express	GIBCO	Cat#12604013
Experimental Models: Cell Lines		
L Wnt-3a cells	ATCC	CRL-2647™; RRID:CVCL_0635
Other		
Elplasia™ plate	Kuraray	Cat#RB 500 400 NA
Ultra-low attachment plates (6-well)	Corning	Cat#3471
Human embryonic stem cell medium	WICELL, 2018	N/A
L Wnt-3A conditioned medium	Fang et al., 2006	N/A
Melanocyte differentiation medium	Fang et al., 2006 , Ohta et al., 2011	N/A

MATERIALS AND EQUIPMENT

The Key Resources Table details all required materials and equipment.

STEP-BY-STEP METHOD DETAILS

iPSC Preparation

⌚ **TIMING:** ~2 weeks

There are two goals for this step:

- a. To select iPSC lines with potential to differentiate into melanocytes,
- b. To guarantee that these iPSCs are in good state before induction.

⚠ **CRITICAL:** Different iPSC lines show different potentials towards melanocyte differentiation. Therefore, it is critical to select or screen proper cell lines for this purpose (see *Before you Begin, Selection of iPSC Lines* above).

⚠ **CRITICAL:** iPSCs that are not properly adjusted to a normal state usually fail to differentiate into melanocytes.

Thaw Mouse Embryonic Fibroblasts (MEFs)

⌚ **TIMING:** ~50 min

1. Add 2 mL 0.1% gelatin to each 60 mm dish and ensure that the solution coats the entire bottom of the well.
2. Incubate at 37°C for at least 30 min.
3. Aspirate the gelatin and discard.
4. Plate mitomycin C-inactivated MEFs (at a density of 6×10^5 viable cells/60 mm dish) and culture them in 5 mL DMEM (high glucose) supplemented with 10% FBS. Incubate MEFs at 37°C for 12–24 h.
5. Check whether the cell density reach at least 70%–80% confluence.

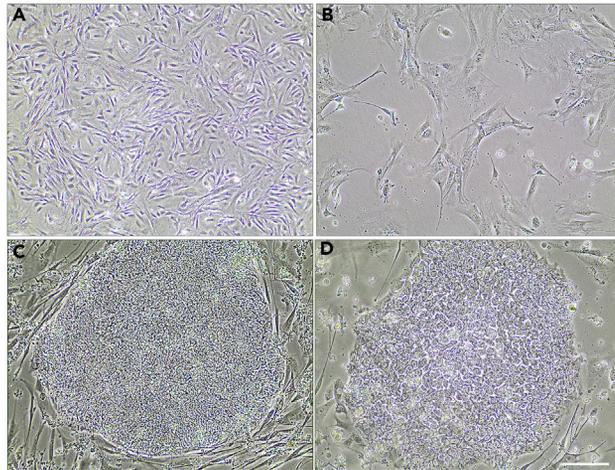


Figure 3. The Quality and Density of MEFs Are Closely Related to the Quality of the iPSCs
Proper density of MEFs with good quality (a) and too low density with bad quality (b, large and flat morphology). Undifferentiated iPSCs typically show a colony with a sharp edge, compact cell junction, and high cell density in the center (c). Colonies with enlarged cells and significant fissures between cells usually indicate spontaneous differentiation (d). Scale bar: 200 μ m.

⚠ **CRITICAL:** Both the quality and density of MEFs are critical for iPSC cultures; poor quality or low density can lead to spontaneous iPSC differentiation (Figure 3).

⚠ **CRITICAL:** Check the quality (as judged by morphology) and determine the percent viability for each batch of mitomycin C-inactivated MEFs before use. If cells are found at a low confluence the day after plating, it is necessary to seed more cells into the original MEF cultures to reach the required 70-80% confluence and use them for iPSC cultures the next day.

Thaw the Frozen iPSCs

⌚ **TIMING:** ~30 min

1. Prepare mitomycin C-treated MEFs the day before thawing the iPSCs (refer to Thaw Mouse Embryonic Fibroblasts).
2. Aspirate the medium and thoroughly wash the MEFs with 5 mL pre-warmed DMEM to remove FBS. Discard DMEM.
3. Add 4 mL hES medium to each 60 mm dish and return to the incubator.
4. Prepare 5 mL DMEM/F12 medium in a 15 mL conical tube.
5. Transfer the cryovial of iPSCs from liquid nitrogen to the 37°C water bath to thaw the cells quickly.
6. Gently pipette (2–3 times) and transfer the cell suspension to the tube prepared in step 4.
7. Add 1 mL DMEM/F12 medium into the frozen tube to collect the residual cells and transfer the suspension into the same conical tube.
8. Centrifuge at 200 \times g for 5 min at 4°C.
9. Discard the supernatant, add 1 mL hES medium and resuspend the cells pipetting 2–3 times gently.
10. Transfer the cell suspension to the dish prepared in step 3 and add Y-27632 to a final concentration of 10 μ M.

11. Gently rock the plate side to side, and back and forth, to achieve an even dispersion of cells across the well and incubate in a 37°C incubator ([Methods Video 1](#)).

▮▮ **PAUSE POINT:** Do not disturb the dish for at least 12 h.

12. Change the medium every day thereafter using MEF-based media (hES medium).

Passage of iPSCs (MEF-Based Conditions)

⌚ **TIMING:** ~40 min

1. Prepare mitomycin C–treated MEFs the day before passage (refer to steps 1–3 in Thaw the Frozen iPSCs).
2. When the iPSCs are of adequate size (usually 0.8–1 mm in diameter), discard the culture medium.
3. Wash the cells once with 4 mL DMEM/F12 medium and discard the wash medium.
4. Add 700 μ L dissociation solution (prepared in Before you Begin, Dissociation Solution for Human iPSC Passage on MEF) and ensure that the solution covers the entire bottom of the well.
5. Incubate at 15–25°C and observe cells under an inverted microscope.
6. When the edges of the iPSC colonies become curled and unclear, remove the dissociation solution completely.
7. Wash cells twice with 4 mL pre-cooled DMEM/F12 medium. Aspirate and discard the wash medium.
8. Add 4 mL DMEM/F12 medium to the dish and gently pipette to break the colonies into small clumps.

⚠ **CRITICAL:** Do not pipette too vigorously; avoid breaking the iPSCs into single cells.

9. Transfer the cell suspension to a 15 mL conical tube and wash the dish with 4 mL DMEM/F12 medium to collect the residual clumps.
10. Centrifuge at 200 \times g for 5 min at 4°C.
11. Discard the supernatant, add hES medium, and gently resuspend the cells.
12. Aliquot the cell suspension (the ratio depends on cell lines) into the dishes prepared in step 1.
13. Cross-shake and culture in a 37°C, 5% CO₂ humidified incubator.

Note: Using pre-cooled media while passaging maintains low cellular metabolic activity thereby reducing cellular damage after detachment.

Passage of iPSCs (Feeder-Free Condition)

⌚ **TIMING:** ~60 min

1. Prepare Matrigel-coated plates (refer to Before you Begin, Matrigel-Coated Plate).
2. Discard the culture medium. Wash the cells once with 2 mL pre-cooled DMEM/F12 medium.
3. Add 600 μ L of ReLeSR, ensuring the solution covers the entire bottom of the well. Aspirate ReLeSR within 1 min.
4. Incubate at 15–25°C and observe cells under an inverted microscope.
5. When there are obvious fissures between cells (about 5–8 min), tap the side of the plate firmly.
6. Add 2 mL mTeSR1 medium to the well and gently pipette to break large aggregates into small clumps.

△ **CRITICAL:** Do not pipette too vigorously; avoid forming clumps that are too small, or single cells.

7. Aliquot the cell suspension into the Matrigel-coated plates with adequate splits (the ratio depends on cell lines) and add additional medium to make the final volume 2 mL in each well.
8. Cross-shake and culture in a 37°C incubator.
9. Change the medium every day.

Alternatives: Depending on lab preference, the two-abovementioned dissociation solutions (dissociation solution for human iPSCs passage on MEF, and ReLeSR™) can be replaced with other commercial reagents, such as TrypLE™ Express, that perform a similar function.

Generation of Embryoid Bodies (EBs)

⌚ **TIMING:** ~1 week

This step generates optimal EBs for melanocyte differentiation. Embryoid bodies can be formed either using aggregates with ultra-low attachment plates or using single cells with micro-space plates.

- a. The aggregate method follows a simpler protocol and is recommended for inexperienced researchers.
- b. The single cell method can generate EBs with uniform size and morphology.

EB Formation using Aggregates

⌚ **TIMING:** ~30 min

1. Dissociate iPSCs cultured under MEF-based conditions to generate small aggregates (refer to steps 1–10 of Passage of iPSCs, MEF-based conditions). Do not pipette vigorously; avoid breaking iPSCs into too small aggregates or single cells.
2. Discard the supernatant, add 2 mL hES medium (without bFGF), and gently resuspend the cells.
3. Transfer these aggregates to ultra-low attachment plates and add Y-27632 to a final concentration of 10 μM.
4. After 24 h, collect the aggregates and transfer them into 15 mL conical tubes.
5. Centrifuge at 200 × g for 30 s at 15–25°C, to deposit the aggregates.
6. Carefully aspirate the supernatant to remove Y-27632.
7. Gently resuspend the aggregates in fresh hES medium (without bFGF) and transfer into the ultra-low attachment plates with 2 mL medium in each well.
8. Change the medium when necessary (usually every day).

Optional: The dissociation solution for EB formation, using aggregates, can be replaced by other commercial reagents such as TrypLE™ Express.

Optional: hES medium (without bFGF) for EB cultures can be replaced by AggreWell™ EB formation medium.

Optional: iPSCs cultured in feeder-free conditions can also be used to generate EB with the above protocol.

EB Formation using Single Cells

⌚ **TIMING:** ~60 min

1. Aspirate the culture medium of iPSCs cultured in feeder-free condition, wash the cells with DMEM/F12 medium and discard.
2. For each well of a 6-well plate, add 600 μL ACCUTASE™.
3. Incubate for 4–6 min at 15–25°C. Check the cell morphology frequently using an inverted microscope.
4. When the cells have become round, remove ACCUTASE™.
5. Add 2 mL of DMEM/F12 and gently pipette the cells to dissociate them into single cells.
6. Transfer the entire suspension to a 15 mL conical tube.
7. Wash the plate with another 2 mL of DMEM/F12 to collect the residual cells and transfer the suspension to the same tube.
8. Centrifuge the cells for 5 min at 300 $\times g$ at 4°C. Discard the supernatant.
9. Resuspend the pellet in 1 mL AggreWell EB formation medium.
10. Determine the viable cell density.
11. Seed 2.5–5 $\times 10^5$ cells into each well of an Elplasia™ plate (24-well), add additional medium until there is a final volume of 2 mL.
12. Add Y-27632 to a final concentration of 10 μM .
13. Cross-shake the plate repeatedly to ensure the cells distribute uniformly across the micro-spaces.
14. Culture in a 37°C incubator.
15. After 24 h, gently pipette the bottom of the Elplasia™ plate and float the aggregates.
16. Collect the aggregates and transfer them into a 15 mL conical tube.
17. Allow the tube to stand for 2–3 min to allow the aggregates to settle.
18. Aspirate the supernatant carefully to remove Y-27632.
19. Gently resuspend the aggregates in fresh AggreWell EB formation medium and transfer into the ultra-low attachment plates, with 2 mL medium in each well.
20. Change the medium when necessary (usually every day).

Note: The frequency of media change depends on the number of EBs in each well and their growth capability.

△ CRITICAL: Medium change for EB formation must be timely and adequate to ensure that there are enough nutrients. Infrequent medium changes will lead to poor status.

△ CRITICAL: EBs in a good state have smooth borders and dark centers without cavities (Figures 2C and 2D), while EBs in a poor state have blurry boundaries and/or break easily and cannot be used for melanocytes differentiation (Figures 2A and 2B).

Note: To ensure a sufficient supply of fresh nutrients, split EBs into multiple wells when necessary.

Note: When EBs reach about 300–500 μm in diameter and have compact centers (usually between 5 and 10 days, depending on the cell line), they are ready for differentiation (Figures 2C and 2D).

Melanocyte Differentiation

⌚ **TIMING:** ~5–6 weeks

This step generates iMelanocytes from EBs; it takes about 5–6 weeks to generate fully differentiated iMelanocytes.

Note: We divide the differentiation procedure into early and late stages, according to the time point, cell growth environment, and medium components.

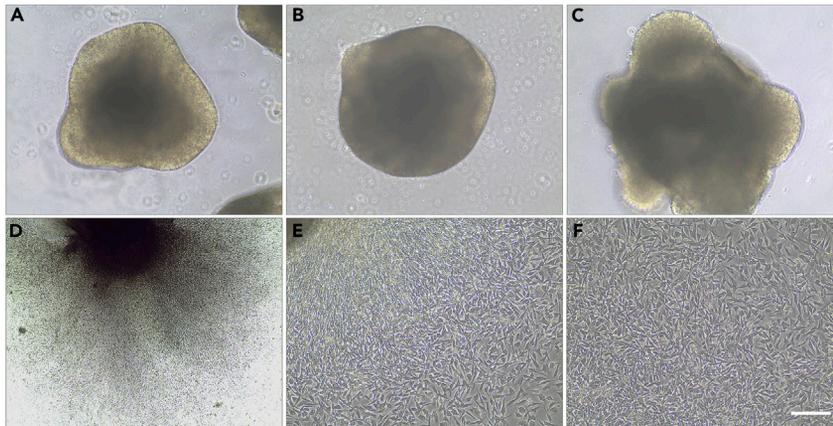


Figure 4. Early Stage of iPSC-Derived Melanocyte Differentiation

During the first two weeks of suspension culture differentiation, EBs enlarge continuously. Although they show various morphologies, smooth and bright boundaries are typically found (week 2, a–c). Large quantities of melanocyte-like cells show a vigorous proliferative ability after they attach to the fibronectin-coated dishes (week 3, d–f). Scale bar: a–c, e, and f, 200 μm ; d, 50 μm .

Early Stage of Differentiation

⌚ TIMING: ~3 weeks

Early stage refers to the differentiation in the first 3 weeks and cells grow in colonies (Figure 4).

1. Collect EBs that are ready for differentiation and transfer them into a 15 mL conical tube.
2. Allow the tube to rest for 2–3 min and allow the aggregates to settle.
3. Carefully remove the supernatant.
4. Resuspend EBs in melanocyte differentiation medium and transfer them into ultra-low attachment plates; for each well of a 6-well plate, 20 EBs are acceptable.
5. Replace half of the medium every 2–3 days.
6. Two weeks later, collect the differentiated EBs and transfer them to fibronectin-coated plates; for each well of a 6-well plate, 10 EBs are acceptable).
7. Continue the differentiation using melanocyte differentiation medium for another 7 days.
8. Replace half of the medium every 2–3 days.

Single Cell Dissociation

⌚ TIMING: ~30 min

At week 3 of differentiation, the density of iMelanocytes is very high (Figures 4D–4F), and they need to be dissociated into single cells and passaged (late stage).

1. Prepare fibronectin-coated plate (refer to Before you Begin, Fibronectin-coated plate) at least 1 h ahead of single cell dissociation.
2. Aspirate and discard the culture medium. Gently wash the cells with DPBS.
3. For each well of a 6-well plate, add 600 μL TrypLE Express Enzyme and ensure complete coverage of cells.
4. Incubate for 2–3 min at 15–25°C. Frequently observe the cell morphology using an inverted microscope.
5. When the differentiated cells become round, add 2 mL of DMEM (low glucose) to dilute the enzyme.

6. Gently pipette the cells and transfer the entire suspension to a 15 mL conical tube.

Note: Undifferentiated cells usually form aggregates after dissociation.

△ **CRITICAL:** do not pipette too vigorously to avoid dissociating them into single cells.

7. Wash the plate with another 2 mL of DMEM to collect the residual cells and add to the initial suspension.
8. Allow the tube to stand for 2–3 min and allow the aggregates to settle.
9. Carefully collect the suspension from the top of the tube and centrifuge for 5 min at 300 × g at 4°C.
10. Discard the supernatant and resuspend the pellet with melanocyte differentiation medium (supplemented with 0.5% FBS and without TPA).
11. Determine the viable cell density.
12. Seed iMelanocytes onto fibronectin-coated plates at a density of $2 \times 10^4/\text{cm}^2$.
13. Change the medium every 2–3 days and passage when the cells reach 90% confluence.

Note: Commercial melanocyte medium, such as HEM 2201, is not suggested for the late stage culture as it cannot maintain iMelanocytes as long as the differentiation medium and usually leads to senescence.

Note: iMelanocytes need to be plated at a relatively higher density ($1.5\text{--}2 \times 10^4/\text{cm}^2$ is recommended) than normal human epidermal melanocytes (usually $1 \times 10^4/\text{cm}^2$), otherwise the proliferative ability decreases.

Passage of iMelanocytes

⌚ **TIMING:** ~30 min

After single cell dissociation, epithelium-like cells and/or undifferentiated cells can initially be observed. Compared to iMelanocytes, it usually takes longer for these cells to become detached. As a result, differential dissociation can be used to purify iMelanocytes.

1. Prepare fibronectin-coated plates (refer to Before you Begin, Fibronectin-Coated Plate) at least 1 h ahead of single cell dissociation.
2. When the cells reach 90% confluence, aspirate and discard the culture medium.
3. Gently wash the cells with DPBS.
4. For 60 mm dishes, add 600 μL TrypLE Express Enzyme and ensure complete coverage of cells.
5. Incubate for 1–2 min at 15–25°C. Keep observing the cell morphology using an inverted microscope.
6. When iMelanocytes form a round shape, gently tap the flask to detach the cells. (The epithelium-like cells and undifferentiated cells usually have not become round at this stage.)
7. Add 2 mL DMEM (low glucose) to dilute the enzyme and avoid pipetting the bottom of the well.
8. Transfer the entire suspension to a 15 mL conical tube.
9. Wash the plate with fresh 2 mL DMEM to collect the residual cells, avoid pipetting the bottom of the well, and combine with the previous suspension.
10. Centrifuge the cells for 5 min at 300 × g at 4°C. Discard the supernatant.
11. Resuspend the pellet with melanocyte differentiation medium (supplemented with 0.5% FBS and without TPA).
12. Determine the viable cell density (ideally > 90%).
13. Transfer the iMelanocytes into newly prepared fibronectin-coated plates at a density of $2 \times 10^4/\text{cm}^2$.

Storage of iMelanocytes

⌚ TIMING: ~30 MIN

This step describes storage of iMelanocytes for transplantation or other applications.

⚠ **CRITICAL:** The quality of iMelanocytes must be confirmed before storage.

⚠ **CRITICAL:** Freezing steps must be followed strictly to ensure success.

1. Dissociate the iMelanocytes (Refer to step 1–9 of Passage of iMelanocytes).
2. Determine the viable cell density.
3. Centrifuge the cells for 5 min at 300 × g at 4°C. Discard the supernatant.
4. Resuspend the pellet with the appropriate volume of freezing medium (90% FBS supplemented with 10% DMSO).
5. Dispense aliquots of cell suspensions into cryovials.
6. Put the vials in a gradient cooling box and store at –80°C.
7. After 24 h, transfer the frozen cells to liquid nitrogen.

⏸ **PAUSE POINT:** iMelanocytes can be stored in liquid nitrogen for at least one year, maintaining a good proliferative capacity after thawing.

Note: Due to the damage caused by freezing, the cell density needs to be a little higher when thawing than when passaging (see also Single Cell Dissociation).

EXPECTED OUTCOMES

Morphology

The most convenient method to identify the generation of iMelanocytes is through observation of cellular morphology. Dendritic cells can be observed when they attach to fibronectin-coated plates at week two (Figure 5A) and the dendrites become more and more typical (Figure 5B), especially after single-cell dissociation (Figure 5C). Healthy iMelanocytes are robust cells as indicated by quick proliferation capability and clear cellular morphology (Figures 4D–4F and 5A–5C) even after single-cell dissociation (Figure 5C). In addition, the color of cell pellet also darkens gradually with passages, becoming notably dark by week 5–6, representing melanocyte maturation.

Melanocyte Markers

Melanocyte markers such as PAX3, SOX10, and MITF are expressed as early as the first week of differentiation, which can be detected by both real-time PCR (Figure 6) and immunofluorescence staining (Figure 7). Expression of these markers steadily increases as differentiation progresses. If the proper iPSC line is identified and their stemness is well maintained, the percentage of PAX3-positive cells can reach $79.5 \pm 13.7\%$ by week two. Significant expression of markers such as TYR, TYRP1, and DCT, is often detected by week three (Figures 6 and 7).

Other Evaluations

DOPA staining and Masson-Fontana staining, can be used to detect the activity of tyrosinase and melanin production, respectively. In addition, the generation of melanosomes can be detected using transmission electron microscopy; however, the procedure is more complicated than the above methods. It is recommended that these evaluations be performed after week 5 of differentiation, when iMelanocytes become fully differentiated and will produce positive results.

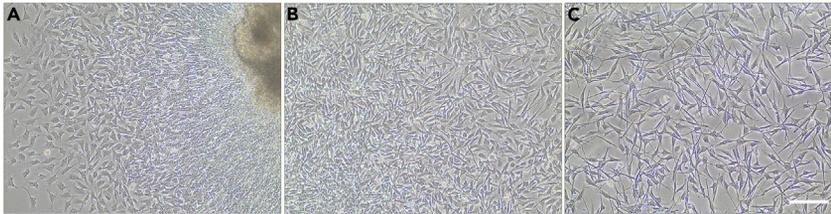


Figure 5. Changes in Cellular Morphology during Differentiation

Cells with short dendrites can be found when they attach to fibronectin-coated plates (week 2, a), and these dendrites become longer one week later (week 3, b) and more typical after single cell dissociation (week 5, c). Scale bar: 200 μm .

LIMITATIONS

Even when all the steps in the above protocol are strictly followed, melanocyte differentiation remains unstable because maintaining the stemness of iPSCs is quite difficult. This is especially true for feeder-free conditions, which requires adequate experience and a good knowledge of cell culture.

The use of iPSCs maintained under feeder-free conditions beyond 20 passages is not recommended (there are no strict limits for this, due to the variability among different iPSC lines). Cultures extended beyond 20 passages must be used with caution. The differentiation capability and karyotype must be checked for cells that are past 20 passages under feeder-free conditions.

This protocol generates a heterogeneous population of melanocytes, with cells maturing at different rates (Liu et al., 2019). The isolation and culture of melanocytic stem cells (MelSCs) are not addressed in our protocol, although these cells can be detected during the differentiation and transplantation in the mouse model. Considering the critical role of MelSCs, identifying

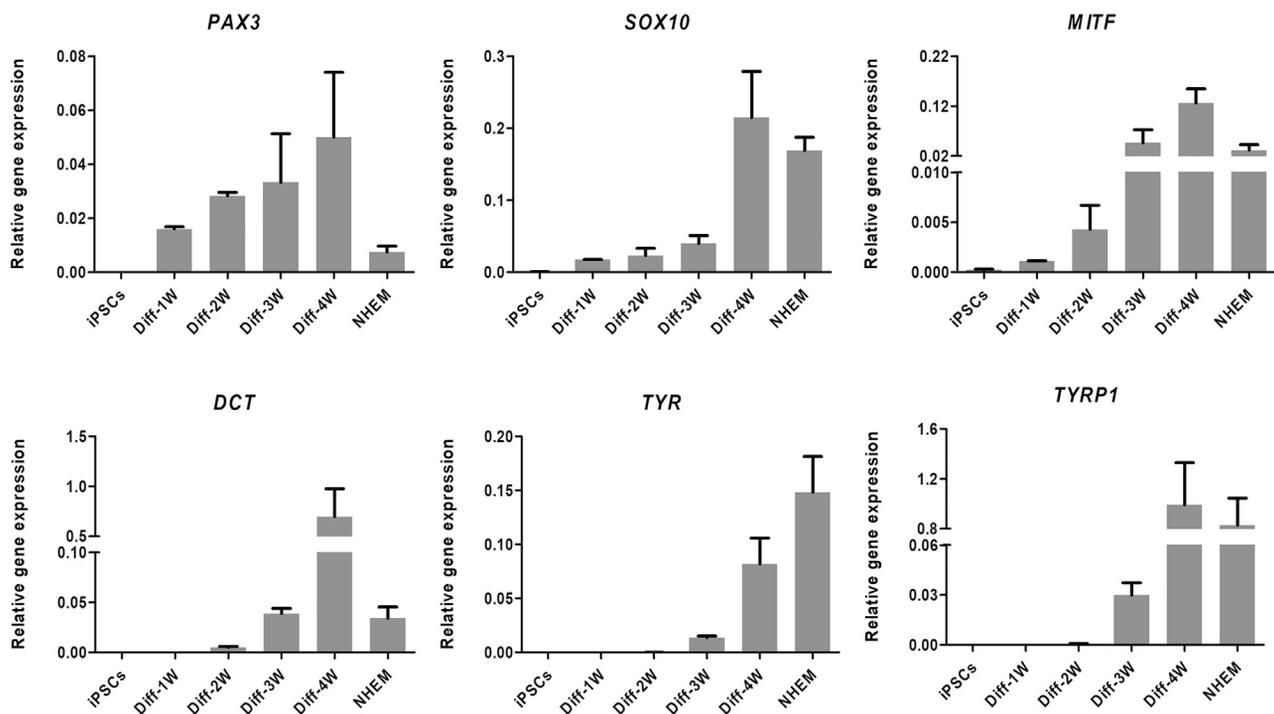


Figure 6. Gene Expression of Melanocyte Markers during Differentiation

The gene expression of melanocyte markers detected by real-time PCR at different time-points during differentiation. NHEMs: normal human epidermal melanocytes; Diff: differentiation; Data are represented as mean \pm SD.

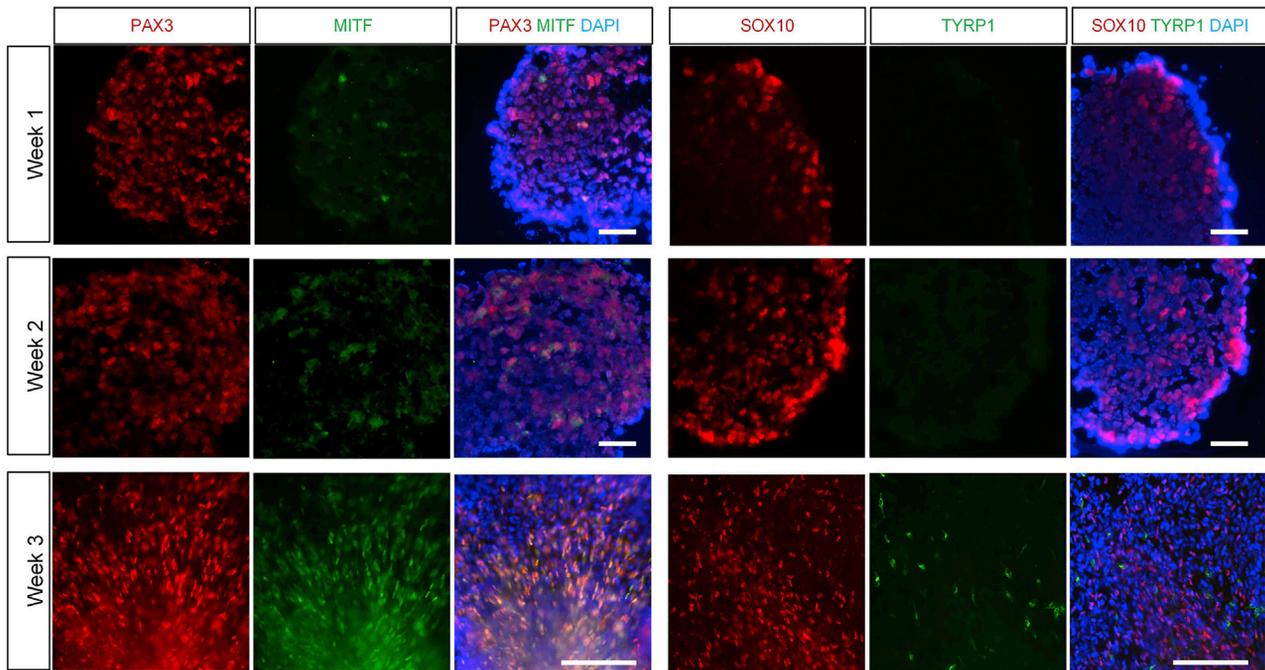


Figure 7. Expression of Melanocyte Markers by Immunofluorescence Staining

PAX3, MITF and SOX10 expression is detected by one week post-differentiation while TYRP1 is not expressed until week three. Scale bar: 100 μ m

markers, isolation procedures and niche regulatory factors may provide a novel potential therapeutic option for patients with pigmentary disorders or hair graying (Lee and Fisher, 2014). Note that the use of alternative brands of feeder-free medium with this protocol has not been tested.

TROUBLESHOOTING

Potential Problem 1

During the suspension culture, EBs fail to form a smooth and bright boundary (Figures 8A–8C). Even though typical melanocyte-like cells are found after attachment to fibronectin-coated plates, massive cell death will occur when they proliferate too quickly. They are characterized by unclear edges, aggregation and a yellow-brown color (Figure 8D, arrows) followed by detachment after 1–2 days (Figures 8E and 8F, arrows).

Potential Solutions

It is critical to choose undifferentiated iPSCs that are well maintained under MEF-based condition or feeder-free condition with a low passage number. If these problems occur, it is better to thaw early-passage iPSCs, which are stored in liquid nitrogen, and start from Section 2 (iPSC PREPARATION) again. For inexperienced researchers, selection of iPSCs maintained on MEFs is recommended.

Potential Problem 2

No significant melanocyte-like cells can be found, or their number is very limited, after attachment to fibronectin-coated plates (Figure 1)

Potential Solutions

Selection or screening of proper cell lines for melanocyte differentiation is important because different iPSC lines show different potentials. If this problem occurs, it is suggested to choose another iPSC line; it is recommended that an established cell line is used, e.g., hES H9, that has confirmed potential for melanocyte differentiation.

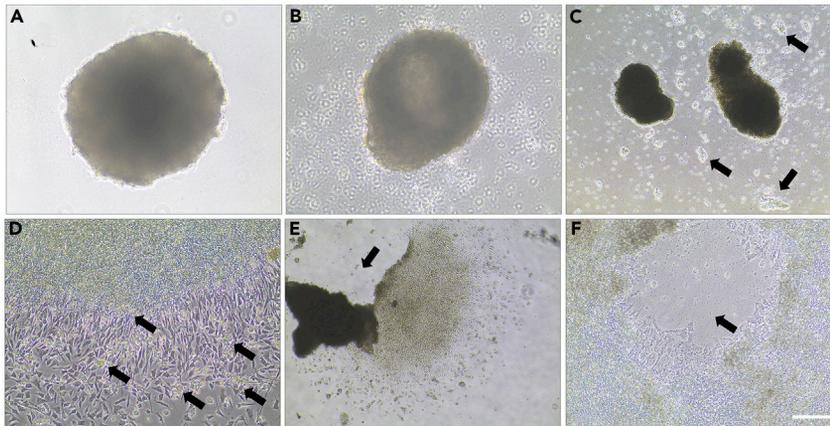


Figure 8. Failure in iMelanocyte Differentiation

EBs become blurry (a), show cavities (b) or break into debris (c, arrows) during the suspension culture differentiation. After attachment at week 2, even typical melanocyte-like cells could be found in some colonies (d), massive cell death will occur which are characterized by unclear edges, aggregation and a yellow-brown color (d, arrows) followed by detachment after 1–2 days (e, f, arrows). Scale bar: 200 μ m.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2019.100004>.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.-W.Z. and L.-P.L.; Investigation, L.-P.L. and N.-N.G.; Resources, Y.-M.L. and Y.-W.Z.; Writing – Original Draft, L.-P.L.; Review & Editing, Y.-W.Z. and L.-P.L.; Supervision, Y.-W.Z. and Y.-M.L.; Funding Acquisition, Y.-W.Z., Y.-M.L., and L.-P.L.

DECLARATION OF INTERESTS

The authors have a patent pending for a suspension system of iPSC-derived melanocyte differentiation: CN201810419157.6 (2018-05-04), PCT/CN2019/093509 (2019-06-28), US16613054 (2019-11-12).

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