Chapter 5

Using Microfluidics to Generate Human Naïve and Primed Pluripotent Stem Cells

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Abstract

Human induced pluripotent stem cells (iPSCs) are generated from somatic cells by the expression of a cocktail of transcription factors, and iPSCs have the capacity to generate in vitro all cell types of the human body. In addition to primed (conventional) iPSCs, several groups recently reported the generation of human naïve iPSCs, which are in a more primitive developmental state and have a broader developmental potential, as shown by their ability to form cells of the placenta. Human iPSCs have broad medical potential but their generation is often time-consuming, not scalable and requires viral vectors or stable genetic manipulations. To overcome such limitations, we developed protocols for high-efficiency generation of either conventional or naïve iPSCs by delivery of messenger RNAs (mRNAs) using a microfluidic system. In this protocol we describe how to produce microfluidic devices, and how to reprogram human somatic cells into naïve and primed iPSCs using these devices. We also describe how to transfer the iPSC colonies from the microfluidic devices over to standard multiwell plates for subsequent expansion of the cultures. Our approach does not require stable genetic modifications, is reproducible and cost-effective, allowing to produce patient-specific iPSCs for cell therapy, disease modeling, and in vitro developmental studies.

Key words Reprogramming, mRNAs, Microfluidics, Pluripotency, Induced pluripotent stem cells

1 Introduction

Human pluripotent stem cells (hPSCs) have been derived from the epiblast of pre-implantation blastocyst as human embryonic stem cells (ESCs) [1] or from somatic cell reprogramming as induced pluripotent stem cells (iPSCs) [2, 3]. Conventional hPSCs molecularly resemble post-implantation epiblast cells in a developmental stage called primed pluripotency. Several primed hPSCs exhibit a strong differentiation bias toward some germ layers [4] and display epigenetic abnormalities and inconsistent X chromosome status [5–7].

Recently, hPSCs in a more primitive developmental state, called naïve pluripotency, have been obtained following the expression of

Peter Rugg-Gunn (ed.), *Human Naïve Pluripotent Stem Cells*, Methods in Molecular Biology, vol. 2416,
https://doi.org/10.1007/978-1-0716-1908-7_5,

transgenes in primed hPSCs [8, 9], directly from human embryos [10] or from somatic cell reprogramming [11–14]. Naïve hPSCs have the unique capacity to differentiate into trophectoderm [15–17] and primitive endoderm [18] allowing the possibility to generate in vitro models of two fundamental extraembryonic tissues, the placenta and the yolk sac, respectively. Furthermore, human naïve iPSCs do not acquire aberrant methylations and they do not display somatic memory of the source cells [7]. Moreover, because female naïve hPSCs contain two active X chromosomes [19], the process of human X inactivation can be elucidated.

To overcome the ethical and technical limitations in deriving human naïve hPSCs directly from the embryo, we derived human iPSCs by somatic cell reprogramming using a transgene-free system of mRNAs combined with a microfluidics approach [11]. This method generates transgene-free primed or naïve iPSCs with higher efficiency and higher throughput compared to the standard reprogramming performed in the classical culture system (multiwell plates or dishes).

Here we describe how to fabricate microfluidic devices, how to reprogram human fibroblasts into either naïve [11] or primed [20, 21] iPSCs using mRNAs, and how to stabilize in culture the iPSCs obtained.

2 Materials

2.1 Microfabrication

1. Propylene glycol monomethyl ether acetate (>99%).
2. Hexamethyldisilazane (>99%).
3. SU-82100 photoresist (MicroChem).
4. 4-inch Silicon Wafer, thickness 525 ± 20 μm, Single Side Polished (Siegert).
5. Spin coater.
6. Vacuum pump.
7. Hot plate.
8. UV light lamp.
9. Glass dish.
10. Shaker.
11. Desiccator.
12. 1-mm and 3-mm biopsy punches.
13. Coverslips, 24 × 60 mm, 0.13–0.17 mm thick.
14. Air plasma cleaner.

15. Base/curing agent mix: Prepare 10 parts of Sylgard 184 base and 1 part of the curing agent by weight (reagents are available from Dow Corning). For example, when a Petri dish of 75 mm in diameter is used, weigh 70 g of the base and 7 g of the curing agent (10:1 ratio) to obtain Polydimethylsiloxan (PDMS) stamps of roughly 10 mm thickness. Mix the two components thoroughly for at least 3 min.
16. MICRO-90 cleaning solution: 2% (v/v) in distilled water.

2.2 Materials for Naïve hPSC Reprogramming

1. Dulbecco's Phosphate Buffered Saline, Modified, with $MgCl_2$ and $CaCl_2$ (PBS).
2. Dulbecco's Phosphate Buffered Saline, Modified, without calcium chloride and magnesium chloride (PBS −/−).
3. Trypsin-EDTA (0.05%).
4. Accutase.
5. TrypLE Select Enzyme (1×), no phenol red.
6. BJ Human fibroblasts (ATCC).
7. Mitotically inactivated mouse embryonic fibroblasts (MEFs; DR4 strain, *see* **Note 1**).
8. Low oxygen incubator (37 °C, CO_2 5%, O_2 5%) (*see* **Note 2**).
9. Fibroblast medium: Supplement DMEM/F12 with 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, and 0.1 mM 2-mercaptoethanol. Store medium at 4 °C for up to 1 month.
10. Naïve reprogramming medium: mix StemMACS ReproBREW XF Basal Medium with 50× StemMACS ReproBREW XF Supplement and store at 4 °C for up to 3 days.
11. Complete naïve reprogramming medium: add 200 ng/mL B18R (Vaccinia Virus B18R Recombinant Protein Carrier-Free; *see* **Note 3**), 5 μM Y-27632 and 1 μM CHIR99021 to naïve reprogramming medium.
12. RSeT medium: add the 5× Supplement to RSeT Basal Medium according to the manufacturer's instructions and store at 4 °C for up to 3 days.
13. Complete RSeT medium: add 200 ng/mL B18R to RSeT Medium.
14. mRNAs for naïve hPSC reprogramming. Resuspend each mRNA (*POU5F1*, *SOX2*, *MYC*, *KLF4*, *NANOG*, with or without *GFP* mRNA, *see* **Note 4**) and mix them following the manufacturer's datasheet, omitting *LIN28A* mRNA (*see* **Note 5**). Resuspend each mRNA at a concentration of 100 ng/μL. The molar ratio among the factors should be 3:1:1:1:1:1 where *POU5F1* is the most abundant. Aliquot the mixed mRNAs in small volumes (5–10 μL) and store them at −80 °C in order to avoid freezing and thawing each mRNA

more than twice. Aliquots can be stored at −20 °C for 1 week. mRNAs are available from Miltenyi (StemMACS™ mRNA Reprogramming Kit), Reprocell (Stemgent StemRNA-NM Reprogramming Kit) or produced in-house as described by Warren and colleagues [22] (*see* **Note 6**).

15. Fibronectin solution: 25 μg/mL human plasma fibronectin in PBS. 12 μL are needed for each channel, so prepare the correct amount of solution that is needed for all channels that will be used.
16. StemMACS mRNA transfection mix: thaw mRNA aliquots on ice and remove the StemMACS mRNA Transfection Kit transfection reagent (TR) and the transfection buffer (TB) from the fridge. Prepare the following mixes in a RNase-free sterile 0.2 mL tube:
    (a) Mix A: 0.37 μL TB + 0.09 μL mRNAs.
    (b) Mix B: 0.44 μL TB + 0.03 μL TR.

    The volumes indicated refer to one channel (*see* **Note 7**). Combine Mix A and Mix B by gently pipetting 4–5 times. Incubate the transfection mix (Mix A + Mix B) a room temperature for 20 min.
17. KSR medium: Supplement DMEM/F12 with 20% KnockOut Serum Replacement (KSR), 2 mM L-Glutamine, 0.1 mM 2-mercaptoethanol, and 1% non-essential amino acids. Store medium at 4 °C for up to 1 month.

2.3 Materials for Primed hPSC Reprogramming

1. Trypsin-EDTA (0.05%).
2. Accutase.
3. TrypLE Select Enzyme (1×), no phenol red.
4. BJ Human fibroblasts (ATCC).
5. Low oxygen incubator (37 °C, CO_2 5%, O_2 5%) (*see* **Note 2**).
6. Fibroblast medium: Supplement DMEM/F12 with 10% FBS, 2 mM L-Glutamine, and 0.1 mM 2-mercaptoethanol.
7. Primed reprogramming medium: Supplement E6 medium (available from commercial suppliers or made in-house according to Chen et al. [23]) with 100 ng/mL FGF2, 5 μM Y-27632, 0.1 μM LSD1i (RN-1), and 1% KSR. The medium can be stored at 4 °C for up to 3 days.
8. mTeSR medium: add the 5× Supplement to mTeSR Basal Medium according to manufacturer's instructions and store at 4 °C.
9. mRNAs for primed hPSC reprogramming. If using the Stemgent StemRNA-NM Reprogramming Kit (Reprocell), mix the OSKMNL not-modified RNA (NM-RNA), EKB NM-RNA (used to reduce interferon response), and NM-microRNAs

(two microRNAs from 302/367 cluster that improve primed reprogramming efficiency) following the manufacturer's datasheet. Alternatively, for mRNAs produced in-house, resuspend each mRNA (*POU5F1*, *SOX2*, *MYC*, *KLF4*, *NANOG*, *LIN28A*, and with or without *GFP* mRNA, *see* **Note 4**) at a concentration of 100 ng/μL. The molar ratio among the factors should be 3:1:1:1:1:1, where *POU5F1* is the most abundant (*see* **Note 8**). It is recommended to aliquot the mixed mRNAs in small volumes (5–10 μL) and store them at −80 °C to avoid freezing and thawing each mRNA more than twice. Aliquots can be stored at −20 °C for 1 week. mRNAs are available from Miltenyi, Reprocell, or produced in-house as described by Warren and colleagues [22] (*see* **Note 6**).

10. Vitronectin solution: 25 μg/mL vitronectin in PBS. 12 μL are needed for each channel, so prepare the correct amount of solution that is needed for all channels that will be used.
11. StemMACS mRNA transfection mix: thaw mRNA aliquots on ice and remove the StemMACS mRNA Transfection Kit transfection reagent (TR) and the transfection buffer (TB) from the fridge. Prepare the following mixes in a RNase-free sterile 0.2 mL tube:
    (a) Mix A: 0.37 μL TB + 0.09 μL mRNAs.
    (b) Mix B: 0.44 μL TB + 0.03 μL TR.

    The volumes indicated refer to one channel (*see* **Note 7**). Combine Mix A and Mix B by gently pipetting 4–5 times. Incubate the transfection mix (Mix A + Mix B) a room temperature for 20 min.
12. Matrigel-coated plates: thaw Growth Factor Reduced Matrigel at 4 °C overnight. Prepare 0.5% Matrigel in cold PBS. Transfer 0.5 mL diluted Matrigel solution per well of a 24-well plate (1 mL for a well of a 12-well plate, 2 mL for a well of a 6-well plate). Incubate plate at room temperature for 1 h or overnight at 4 °C. The plate may be used immediately or stored at 4 °C (Matrigel-coated plates can be kept for at least 2–3 weeks).
13. EDTA solution: 0.5 mM of EDTA in PBS−/−.

3 Methods

3.1 Production of Microfluidic Devices

3.1.1 Photomask Fabrication

1. Design the photomask using CAD, Illustrator Software, or Adobe Freehand. The photomask file we used is available upon request.
2. Print the photomask on a transparency film in high-resolution (>2400 dpi).

3.1.2 Master Fabrication Using Photolithography

1. Photolithography is often conducted in a clean room facility (*see* **Note 9**).
2. Wash a silicon wafer with 2-Propanol, followed by rinsing with distilled water; dry the wafer on a hot plate at 120 °C for 30 min.
3. Place the wafer on the chuck of the spin coater and power the vacuum pump (Fig. 1a).
4. Dispense 1 mL of photoresist for each inch of wafer diameter (Fig. 1b).
5. To have a photoresist thickness of 200 μm, spin at 500 r.p.m. for 10 s with acceleration of 100 r.p.m./s and at 1500 r.p.m. for 30 s with acceleration of 300 r.p.m./s.
6. Bake the coated wafer on a hot plate at 65 °C for 5 min and at 95 °C for 30 min.
7. Place the photomask on top of the coated wafer and expose to UV light with an energy of 310 mJ/cm^2 (Fig.1c).
8. Bake the coated wafer on a hot plate at 65 °C for 5 min and at 95 °C for 10 min.
9. Place the wafer in a glass dish and put it on a shaker.
10. Pour propylene glycol monomethyl ether acetate in the glass and shake for 15 min (Fig. 1d).
11. Wash wafer with 2-Propanol.
12. Dry the wafer with compressed air.
13. Bake the coated wafer on a hot plate at 80 °C for 1 h (Fig. 1e).

3.1.3 Fabrication of the Elastomeric Stamp

1. Place the patterned wafer in a Petri dish of 15 cm in diameter and then in a desiccator that contains a small beaker with 100 μL of hexamethyldisilazane.
2. Connect the desiccator to the vacuum line to reduce the pressure inside the desiccator; keep the wafer under vacuum for 30 min.
3. Fill a plastic cup with base/curing agent mix.
4. Place the uncured PDMS in a desiccator and connect it to the vacuum line to evacuate the air; turn off the vacuum and keep the PDMS under vacuum in order to remove the bubbles. After 15 min vent the desiccator.
5. Place the patterned wafer in a Petri dish of 15 cm and pour the base/curing agent mix (with trapped bubbles) to the desired thickness (4–6 mm) (Fig. 1f); remove the bubbles by placing the Petri dish in a desiccator and connect to the vacuum line of the fume hood, to avoid contaminants spread in the air, for 15 min or until most of the bubbles disappear; and turn off the vacuum and vent the desiccator.

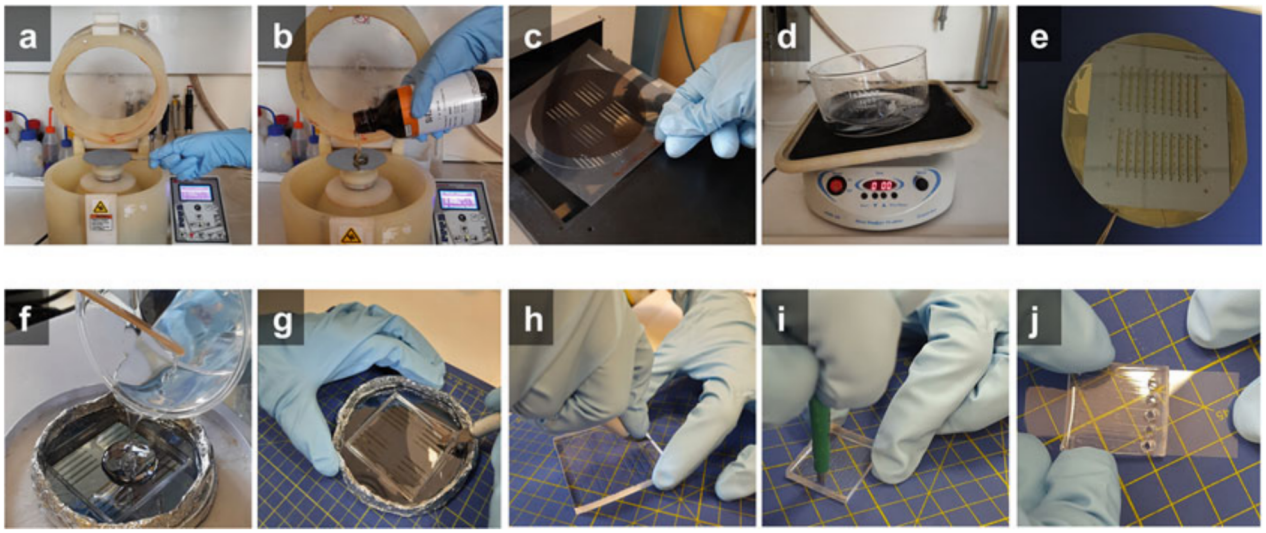

Fig. 1 Soft-lithography process for microfluidic device fabrication. (**a**, **b**) Photo-resit coating on a silicon substrate; (**c**) Exposure of the photoresist to the UV light through the photomask; (**d**) Development; (**e**) Master device after the development; (**f**) Fabrication of the PDMS stamp by using replica molding; (**g**) Detachment of the cured PDMS to the master device; (**h**, **i**) Cutting and punching of the PDMS stamp; (**j**) Bonding between PDMS stamp and the glass surface is the assembly of the microfluidic device after oxygen plasma cleaner treatment

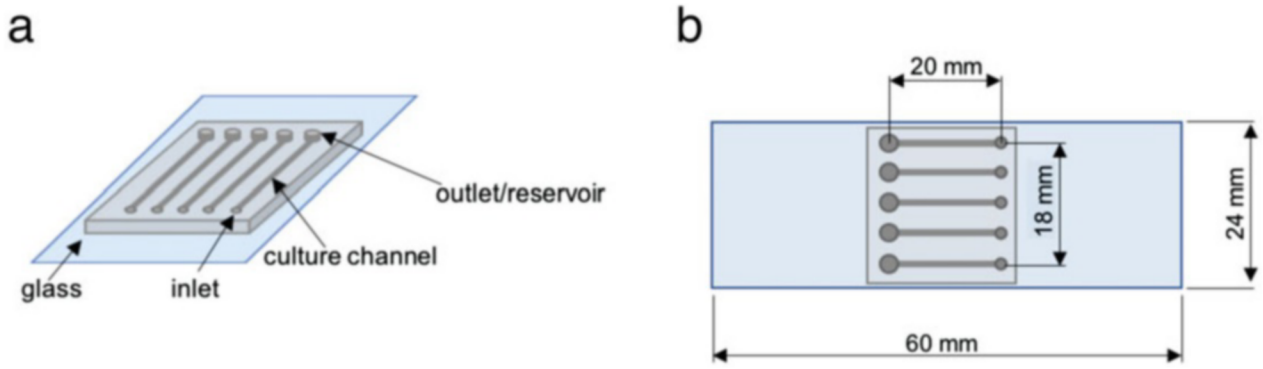

Fig. 2 The microfluidic device. (**a**) Schematic drawing of 5-channel microfluidic device. The microfluidic device is composed by an inlet, a culture channel, and an outlet/reservoir. (**b**) Dimensional details of 5-channel microfluidic device

6. Place the Petri dish containing both the wafer and PDMS in an oven, cure it at 70 °C for 1.5 h.
7. Separate the cured PDMS block from the wafer (Fig. 1g).
8. With the patterned side facing up, use a scalpel to cut the PDMS disk into stamps with the desired dimensions, according to the pattern of the wafer (Fig. 1h).
9. Use a disposable 1-mm biopsy punch to produce inlet holes and a 3-mm punch for outlet holes for each culture chamber (Figs. 1i and 2a, b).
10. Clean thoroughly a microscope glass slide with MICRO-90 cleaning solution and rinse with distilled water.

11. Place the PDMS replica, with the patterned surface facing up, and a glass slide in an air plasma cleaner for surface activation at 0.3 mbar of filtered air and 30 W for 2 min.
12. Place the patterned surfaces of PDMS and the activated glass slide surfaces in direct contact to form the microfluidic device (Fig. 1j).
13. Place the microfluidic device in an oven at 80 °C for 1 h to complete the bonding process.
14. Cool the bonded microfluidic device to room temperature.
15. Pipette 12 μL of 2-propanol inside each culture chamber to clean the chamber from production process by-products, and rinse by flowing 30 μL of distilled water into the chambers before 2-propanol evaporation.
16. Dry the chambers by aspirating the water with a 200 μL pipette and package the microfluidic device inside an autoclave bag.
17. Autoclave at 121 °C for 15 min and leave to dry.
18. Keep the device sterile and in a clear environment until use, for a maximum of 6 months.

3.2 Naïve hPSC Reprogramming

3.2.1 Thawing Human Fibroblasts

1. Prepare a 15 mL tube with 10 mL of fibroblast medium.
2. Thaw a cryovial of human fibroblast cells (*see* **Note 10**) in a thermostatic bath at 37 °C and put the thawed suspension of cells in the 15 mL tube with 10 mL of fibroblast medium. Centrifuge at 300 × *g* for 3 min.
3. Aspirate medium and resuspend cells in fibroblast medium and transfer to cell culture plates.

3.2.2 Passaging and Culture of Human Fibroblasts

1. Warm TrypLE, PBS −/− and fibroblast medium at 37 °C.
2. Aspirate medium from cell culture plate and wash the cells with PBS −/−.
3. Detach cells as single cells by adding the following amount of TrypLE:
   (a) 0.3 mL per well of 12-well plate;
   (b) 0.6 mL per well of 6-well plate.

   Incubate at room temperature for 3–4 min.
4. After 3–4 min of incubation, check cells at the microscope to determine if they are detached as single cells.
5. Gently wash cells with 0.7 mL (for a 12-well plate, use 1.4 mL for a 6-well plate) of fibroblast medium, pipette gently and collect cells into a sterile 2 mL tube. Centrifuge at 300 × *g* for 3 min.
6. Aspirate medium and resuspend cells in fibroblast medium.

7. Plate cells in appropriate volume of fibroblast medium (2 mL for a 6-well plate) and passage cells at a ratio of 1:3 to 1:5. Transfer to incubator.

3.2.3 Day 0, at 3 PM: Seeding Human Fibroblasts into Microfluidic Devices (See Fig. 3a for the Experimental Scheme)

1. Place the microfluidic device inside a 100 mm cell culture dish.
2. Add 3 mL of PBS into the dish around the microfluidic device in order to create a sterile humidified chamber. During the reprogramming protocol, top up the PBS when needed to maintain the humidity (*see* **Note 11**).
3. Pipette 12 μL of fibronectin solution to each channel that will be used. Incubate for 1 h at room temperature.
4. Calculate the number of fibroblast cells that are needed for the reprogramming experiment. Each channel requires 1485 cells (*see* **Note 12**).
5. Detach fibroblast cells from the tissue culture well as follows: wash cells with PBS −/−, add 600 μL of trypsin (for a well of a 6-well plate) and incubate at 37 °C for 4 min, then add 3 volumes of fibroblast medium and transfer the cell suspension into a sterile 15 mL tube.
6. Count the fibroblasts using a Bürker Chamber or automated cell counter and transfer the number of fibroblasts necessary for the experiment into a new sterile 1.5 mL tube. Centrifuge the cells at 300 × *g* for 3 min.
7. Aspirate medium and resuspend cells in fibroblast medium. For each microfluidic channel, 12 μL of volume are needed (which corresponds to ~124 cells per μL).
8. Pipette the 12 μL of cell suspension into each channel.
9. Aspirate the excess liquid from reservoirs (Fig. 2a) with a p200 pipette (but leave the amount of media necessary to form the meniscus).
10. Place overnight in an incubator.

3.2.4 Day 1, at 9 AM: Preparation for the First Transfection

1. Prepare the total volume of complete naïve reprogramming medium needed for the first 3 days of reprogramming.
2. Pipette 12 μL of complete naïve reprogramming medium to each channel and remove the exhausted medium from reservoirs.
3. Put the microfluidic device back into the incubator for at least 1 h before the first transfection.

3.2.5 Day 1, at 10 AM: Preparation of mRNA Transfection Mix and Transfection

1. Add the combined StemMACS mRNA transfection mix into a sterile 0.2 mL tube containing complete naïve reprogramming medium and mix gently 4–5 times. The precise volumes are indicated in Table 1 and they refer to one channel.

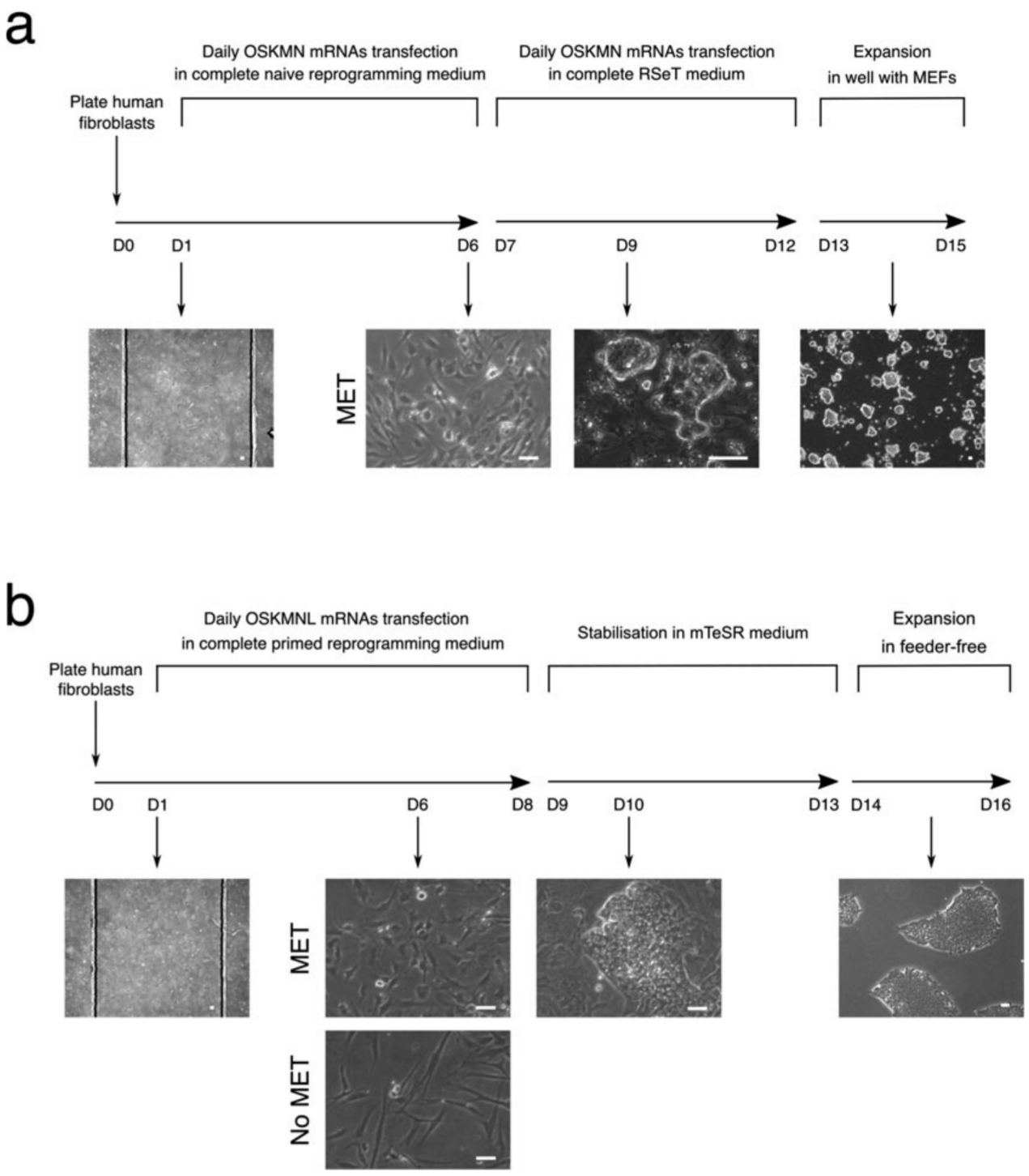

Fig. 3 Naïve and primed hPSC reprogramming. (**a**) Experimental scheme of naïve hPSC reprogramming. On day 1, a representative image of ideal fibroblast seeding density for naïve hPSC reprogramming is shown. On day 6, a representative image of ideal morphology of cells that are undergoing MET is shown. Primary colonies emerge around day 9, which are stabilized as naïve cell cultures in MEF-coated well plate. Scale bars: 50 μm. (**b**) Experimental scheme of primed hPSC reprogramming. On day 1, the ideal fibroblast seeding density for primed hPSC reprogramming is shown. On day 6, representative images show ideal morphology of cells that are undergoing MET and a representative image of cells that failed to perform MET. At day 10, primary colonies emerge, which are stabilized as primed cell cultures in Matrigel-coated well plate. Scale bars: 50 μm