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Microfluidic reprogramming to pluripotency of human somatic cells

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Human induced pluripotent stem cells (hiPSCs) have a number of potential applications in stem cell biology and regenerative medicine, including precision medicine. However, their potential clinical application is hampered by the low efficiency, high costs, and heavy workload of the reprogramming process. Here we describe a protocol to reprogram human somatic cells to hiPSCs with high efficiency in 15 d using microfluidics. We successfully downscaled an 8-d protocol based on daily transfections of mRNA encoding for reprogramming factors and immune evasion proteins. Using this protocol, we obtain hiPSC colonies (up to 160 ± 20 mean ± s.d ($n = 48$)) in a single 27-mm^2 microfluidic chamber) 15 d after seeding ~1,500 cells per independent chamber and under xeno-free defined conditions. Only ~20 μL of medium is required per day. The hiPSC colonies extracted from the microfluidic chamber do not require further stabilization because of the short lifetime of mRNA. The high success rate of reprogramming in microfluidics, under completely defined conditions, enables hundreds of cells to be simultaneously reprogrammed, with an ~100-fold reduction in costs of raw materials compared to those for standard multiwell culture conditions. This system also enables the generation of hiPSCs suitable for clinical translation or further research into the reprogramming process.

Introduction

The discovery that adult human somatic cells can be reprogrammed to pluripotency[1] has enabled previously inaccessible processes of human biology to be studied in vitro, and new personalized biomedical applications to be envisioned in vivo.

Somatic cell reprogramming can be induced by the forced expression of *POU5F1* (*OCT4*), *SOX2*, *KLF4* and *C-MYC* (OSKM; known as Yamanaka's factors)[1]. In the initial stage of reprogramming, the exogenous expression of these transcription factors perturbs the transcriptional network of somatic cells. In response, cells integrate intrinsic and extrinsic cues to remodel chromatin and reach a new epigenetic state. The latter stages of reprogramming, maturation and stabilization, result in the endogenous expression of the core circuitry of pluripotency.

We aimed to develop a protocol for the cost-effective production of quality-controlled human pluripotent stem cells in non-integrating and chemically defined xeno-free culture conditions. The method we developed enables reprogramming of a limited number of human somatic cells with high efficiency, high throughput, and low costs to produce hiPSCs without genomic integration[2]. We achieved these results by downscaling mRNA-based cell reprogramming, using microfluidics to miniaturize the system. Here we describe in detail how to set up and use our miniaturized system.

Development of the protocol

Miniaturization results in an ~50-fold increase of efficiency compared with that of conventional culture systems, even under defined xeno-free conditions[2]. Miniaturization provides two substantial advantages: a notable improvement in mRNA delivery, as a consequence of the short distance between the synthetic mRNA and the cells[2]; and a faster accumulation of cellular extrinsic endogenous factors that positively feed back on cellular reprogramming (Fig. 1). This self-regulation of the cellular microenvironment promotes the conversion of fibroblasts to hiPSC colonies only when a critical ratio between cell density and medium height is used[2].

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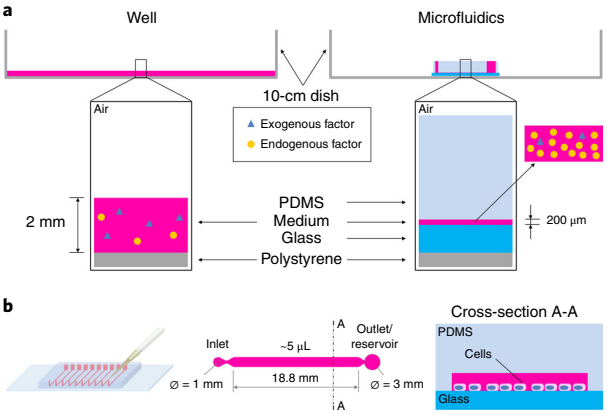

Fig. 1 | Microfluidics downscale and setup. a, Schematic representation of medium reduction and endogenous factor accumulation from a standard well culture system to microfluidics. Endogenous and exogenous factor sizes are not to scale. **b**, Drawing of a ten-chamber microfluidic device during medium change by manual pipetting, and dimensions of a single channel-shaped microfluidic culture chamber. Cell size is not to scale.

This protocol utilizes the advantages of mRNA reprogramming in standard systems, such as the rapidity of exogenous mRNA clearance due to the ~24-h life span of mRNA in the cells[3]. In microfluidics, after only 15 d of reprogramming, a high number of hiPSC colonies are obtained (up to 160 ± 20 mean ± s.d (n = 48)) hiPSC colonies per culture chamber). These colonies can then be extracted from the setup and used for other applications.

The miniaturization of this protocol also offers specific advantages. First, a low number of cells is required (~1,600 cells per chamber). Thus, this protocol can be used when there is limited availabilityv of biological samples, for example, when using primary cells or when cell expansion and cell senescence prevent successful reprogramming. Second, in a single microfluidic device, ten independent parallel experiments can be performed, greatly increasing the experimental throughput. A single operator can easily manage 100 parallel reprogramming experiments with a normal workload. Third, the small volume of the system (~5 µL) reduces the cost of reagents >100-fold and has a considerable effect on the feasibility of reprogramming on a large scale. Many conditions of reprogramming can be tested for a single cell line to increase the reprogramming success rate, as discussed below; somatic cells from large cohorts of patients can be affordably reprogrammed; and multiparametric optimization becomes possible for the development of integrated processes of reprogramming and differentiation[2,4–6].

The protocol we describe here has been improved compared with that used in our original publication[2]. Previously, the reprogramming was based on a daily transfection of modified mRNAs (*OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, *LIN28* (OSKMNL)) for at least 12 d. Moreover, the transfections were coupled with two changes per day of medium supplemented with B18R protein, to reduce interferon response. Medium was freshly prepared each day, and reprogramming was performed in normoxia. This protocol was highly effective but required a heavy workload during the entire duration of the reprogramming, which could last up to 15 d. Thus, we further optimized the protocol to reduce its complexity in terms of medium preparation and to reduce the overall number of medium changes and transfections, while maintaining the high reprogramming efficiency.

The improved version of the protocol we describe here enables microfluidic reprogramming based on commercially available nonmodified mRNAs (NM-RNAs) of the same reprogramming factors (OSKMNL) combined with immune evasion NM-RNAs encoding E3, K3 and B18R protein (EKB), and two microRNAs (miRNAs) from the 302/367 cluster, previously reported in a published protocol[7]. The reprograming process is performed under hypoxia (5% (vol/vol) O_2), an established favorable factor to improve reprogramming efficiency[8]. To efficiently downscale the reprogramming in the microfluidic system, we limited the number of daily transfections to eight for the entire reprogramming, with a single medium change per day.

Moreover, we also adapted the reprogramming protocol to use chemically defined medium (E7 medium). E7 has the same components present in E8[9] (the widely used medium for hiPSC expansion), except that TGFβ is absent. It has a disclosed and simple composition, which makes it suitable

for defined biological studies of cell reprogramming. Finally, we used a new method, based on the use of EDTA as a detachment agent, to selectively extract hiPSC colonies from microfluidic chambers.

Application of the protocol

Microfluidic reprogramming can be adapted to a wide range of applications. There are no major limitations in downscaling other reprogramming methods to the microfluidic system after optimizing the conditions. Moreover, given the feasibility of the production of customized mRNAs by common biological laboratories, the coupling of microfluidic and mRNA technologies provides great flexibility for other applications.

As an example, we performed fibroblast reprogramming in microfluidics also, using a different approach based on self-replicating RNAs of only four reprogramming factors, OCT4, KLF4, SOX2 and GLIS1. The results in Supplementary Fig. 1 show that cells can also be reprogrammed within this setup with only three Yamanaka factors and without c-MYC, with a consistent success rate in 20 independent microfluidic chambers.

Comparison with other methods

In recent years, numerous methods have been developed to generate hiPSCs, with the aim of increasing the efficiency of reprogrammed cells and also enabling the generation of footprint-free hiPSC lines that lack the integration of any viral vector sequences in their genomes. Replication-competent Sendai RNA virus carrying the Yamanaka factors (*OCT4*, *SOX2*, *KLF4*, *c-MYC*) is widely used for skin fibroblast reprogramming[10]. It is relatively efficient, is highly reliable and has a low workload. However, the Sendai virus method does not allow flexibility in the expression level and stoichiometry of the reprogramming factors, and clearance of Sendai virus is relatively slow (>10 passages[11,12]). The substantial amount of work required to clear virus from the hiPSCs during expansion results in high costs for hiPSC colony stabilization. Episomal plasmids, which can be easily produced by single biological laboratories and provide process flexibility[13], have been shown to have lower efficiency than Sendai virus, and the generated hiPSCs retain episomal plasmid sequences at high passages (>10) and have a high aneuploidy rate. Alternatively, a self-replicating RNA-based method has also been proposed[14], but the complete clearance dynamics of the exogenous RNA, which is removed from the cells by the innate immune response, has not been investigated in a comparative study. These features discourage the use of these methods for clinical applications, which require hiPSCs of high quality.

The use of mRNA technology for reprogramming[15,16] has advantages over other non-integrating reprogramming methods. Efficiency is up to 4%, and colonies emerge faster than with other methods. The aneuploidy rate is low, and there is a complete absence of cellular integration after fewer than 5 passages[11]. Moreover, mRNA can be easily produced by biological laboratories, which increases the flexibility of mRNA mix compositions, stoichiometry and level of expression. Specialized companies have already achieved clinical-grade mRNA transfection systems. As also reported in a recent benchmark study that compared the pros and cons of non-integrating methods[11], reprogramming with mRNAs is the most promising approach to fully exploit the potential of hiPSC technology in clinical applications and research settings.

All methods for hiPSC derivation based on conventional culture systems (either Petri dish or multiwell) have considerably high costs, and this can be a limiting factor for many laboratories, especially when multiple patient samples need to be reprogrammed. For example, among the most popular integration-free methods, Sendai virus and mRNA approaches require expensive reagents for reprogramming. Moreover, high labor costs are associated with these methods, owing to the intensive expansion of either starting cells, used with low-efficiency methods, or hiPSCs, for which dilution and clearance of exogenous materials are necessary. For all these reasons, many researchers are limited to small numbers of simultaneous reprogramming experiments, which are often not sufficient to satisfy the need for multiple controls and multiple subject samples. For example, a six-sample hiPSC project, including three controls and three samples from affected subjects, costs more than €10,000 in reagents and requires one full-time researcher to generate hiPSCs. Characterization and clone selection further increase costs. In the best scenario, a recent comparative study showed that the highest efficiency of nonintegrating methods is ~4% when mRNA is used[11].

Microfluidic reprogramming enables large-scale and high-efficiency production of high-quality hiPSCs. Microfluidic technology substantially decreases the cost of reagents and provides the highest efficiency among reprogramming methods. Using microfluidic reprogramming, we were able to

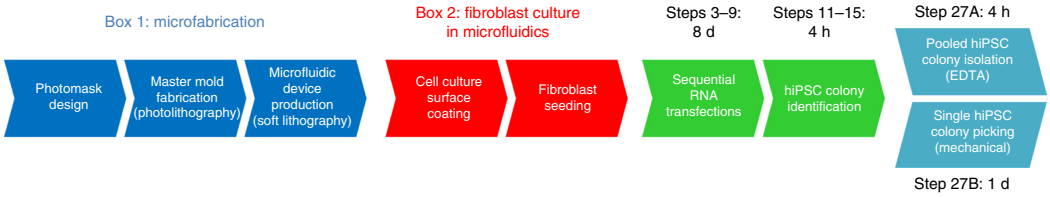

Fig. 2 | Main stages of the protocol.

generate an average number of 6 colonies/mm^2 in 15 d, which is higher than the yields of other methods reported in the literature, which range between 0.1 and 4 colonies/mm^2 in six-well plates[17]. Microfluidic reprogramming requires the seeding of a low number of cells, yet offers a high probability of obtaining colonies. Reprogramming-resistant cells can also be reprogrammed[2]. In addition, the cost of reagents can be decreased by ~100-fold, as microfluidic platforms require only a few microliters of medium (a single chamber is ~5 µL), and only a few nanograms of mRNA is required per day, compared to almost 2 µg when using standard conditions. Last, microfluidics is amenable to automation, offering the possibility of manipulating the microenvironment in a high-precision manner with remote control, thereby strongly reducing the workload and increasing the experimental throughput. Automation is important for large-scale production under strictly defined and clinical-grade conditions. Recently, consortia such as the Human Induced Pluripotent Stem Cells Initiative (HipSci; http://www.hipsci.org) and the iPSC Repository of the California Institute for Regenerative Medicine (CIRM; https://www.cirm.ca.gov/researchers/ipsc-repository) have been formed to increase the availability of hiPSC lines. Cost reduction coupled with system automation make it possible to perform hundreds of parallel reprogramming experiments with different cell sources. Currently, even in manual mode, a single operator can easily manage 100 samples in parallel, using greatly reduced amounts of reagents.

Limitations

Reprogramming of different cell lines results in different success rates. This is a recognized problem in the field, and we have also experienced this limitation[2]. These differences can be ascribed to differences in primary fibroblast isolation protocols, fibroblast senescence and tissue of origin, and patient age and other patient-specific factors. Although understanding the origin of this variability is still incomplete, and the absolute reprogramming efficiency is quite variable, we found that microfluidics shows a great improvement in both success rate and number of colonies per cell seeded as compared with standard well culture systems. In the Troubleshooting section, adaptations of the protocol to increase the success rate on the basis of specific cell line behavior are discussed, but we recommend running different reprogramming conditions in parallel.

The small number of cells that can be cultured on each microfluidic chamber surface (whose area is typically <30 mm^2) could be limiting for methods of analysis that require a high number of cells, such as transcriptomic and proteomic characterizations. Pooled samples and the use of single-cell technologies can overcome this limitation.

mRNA reprogramming is labor intensive because of the need for daily transfections. However, there is a quick clearance of exogenous materials from the obtained hiPSCs. Using already-developed automation could further assist in protocol development and increase throughput[18].

Experimental design

The workflow shown in Fig. 2 describes the main stages of the protocol (Boxes 1 and 2 and Steps 3–27). Four main tasks need to be undertaken, as discussed below.

Microfabrication (Step 1, Box 1)

Box 1 details the procedure for microfluidic device fabrication. A 'mask design' is provided in the Supplementary Manual, whose layout is shown in Supplementary Fig. 2. Thus, a design stage is not needed, unless modifications to the proposed configuration are required. The 'master mold fabrication' is performed according to standard photolithographic techniques. It needs to be performed only once, as the master mold can be reused several times. If facilities are not available, it is advisable to outsource this work, providing an external company with the photomask. 'Microfluidic device

production' refers to the standard soft-lithographic techniques used for the production of the microfluidic devices, called chips, made of polydimethylsiloxane (PDMS), and their attachment to a glass slide to build the final device. This step can be outsourced. Companies such as Onyel Biotech (Italy), or any other company that sells PDMS chips for long-term cell culture, can provide the microfluidic chip according to the protocol described in Box 1. We refer the reader to Box 1 and a previous protocol[19] for instructions on how to perform these first three parts of the current protocol. Final sterilization of the microfluidic devices can be performed by standard autoclaving.

Fibroblast culture in microfluidics (Step 2, Box 2)

In Step 2 and Box 2, we provide advice on how to perform cell culture within microfluidic culture chambers. Before proceeding with a reprogramming experiment, we suggest following the protocol given in Box 2 to ensure that users become familiar with microfluidic technology and to verify the chip functionality. In particular, we describe how to perform the coating of the culture surface and how to change media. By following the explanations in Fig. 3 and Supplementary Video 1, one can carry out microfluidic culture efficiently, without bubble formation or evaporation of the medium.

Sequential RNA transfections (Steps 3–9)

This part of the protocol includes daily transfections of mRNA mix for a period of 8 d. After 8 d, the medium is changed to pluripotency medium for hiPSC colony stabilization and growth for another ~7–9 d. The RNA mix of the reprogramming kit used in this protocol includes three components[7]: NM-RNA of the four Yamanaka factors (*OCT4*, *SOX2*, *KLF4*, *c-MYC*), plus *NANOG*, and *LIN28*; NM-RNA encoding E3, K3, and B18R for evasion of the immune response and to overcome RNA-mediated toxicity; and double-stranded miRNAs from the 302/367 cluster. An increasing dose of the RNA mix is used in transfections from day 1 to day 8 to keep the transfected factor expression in the growing cell population high while avoiding toxicity. The overall goal is to maintain approximately constant amounts of RNA transfected per cell.

In the 'Quality control of main reagents' section, we give suggestions for checking that reagents are working and that cells are properly transfected. This could also be useful in investigations of possible differences in the performance of critical reagents as a consequence of batch variation.

Identification (Steps 11–15) and extraction of the hiPSC colonies (Step 27)

After progressive morphological changes that occur during reprogramming, at day 14 the presence of TRA-1-60^{+} hiPSC colonies can be verified by live staining, and the reprogramming efficiency can be quantified (Fig. 4). Colonies need to grow for an additional 2 d to become larger before isolation.

Depending on the specific application, the colonies formed in a microfluidic chamber can be isolated as a pool or as mechanically picked single colonies (Fig. 5 and Supplementary Video 2), and replated in standard wells for expansion[9] and characterization[20]. The first method of passaging, based on EDTA, is less time-consuming but pools together all the colonies from a single culture chamber. Mechanical picking is more time-consuming and contaminates the hiPSCs with some fibroblasts (which can be removed at subsequent passages), but allows a single colony to expand. The success rate of replating the picked colonies depends on multiple factors, such as the size of cell clusters and the coating of the destination dish, as in standard hiPSC passaging.

Level of expertise

Microfluidic device production requires the use of photo- and soft-lithographic techniques, as well as equipment not commonly available in a biological laboratory, but it can be easily outsourced. However, if the equipment is available, a graduate student can master these skills after 6 weeks of training (Box 1). Training in microfluidic cell culture, described in Box 2, requires one to three experiments to develop adequate competence, and does not necessitate prior knowledge of micro-scale technologies. For the fibroblast reprogramming protocol (Steps 1–27), a biological background with cell culture experience is sufficient. For characterization and passaging of the obtained hiPSC colonies, following established methods that have been described previously[9,20], pluripotent stem cell biology skills are required.

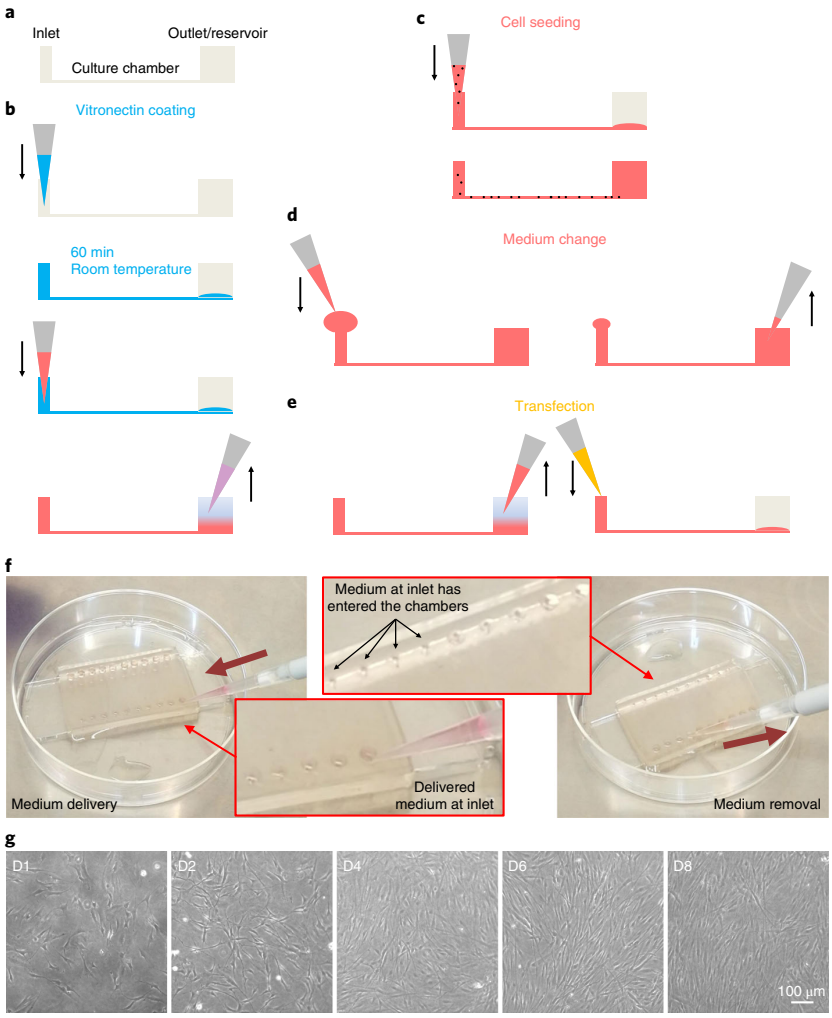

Fig. 3 | Schematic description of the main steps for liquid handling in the microfluidic chip and images of the cell morphology that should be present. a, A longitudinal vertical section of a microfluidic chamber is shown, highlighting the different parts. **b**, Vitronectin coating is applied when the culture chamber is still empty. To ease the inflow of protein solution, the pipette tip should go precisely inside the inlet hole. A volume of 12 µL is sufficient to fill the chamber and avoid bubble formation in the chamber due to evaporation during the 60-min incubation at room temperature. At the end of the incubation period, the protein solution is replaced with fresh medium, which is left in the chamber until cell seeding, usually within an hour. Part of the solution in the reservoir, containing a mixture of Vitronectin solution and medium, is aspirated, without completely emptying the reservoir. **c**, During cell seeding, the pipette tip should be placed precisely inside the inlet hole. 12 µL of well-mixed cell suspension should be injected by quick pipetting. The reservoir will be full at the end of seeding, and it is possible that a small amount of medium will be left at the inlet; it is convenient to avoid aspirating this medium, to obtain spatially homogeneous seeding along the culture chamber. **d**, During medium changes in the next days, the strategy that minimizes bubble formation and time consists of injecting 12 µL of medium near the inlet; because the reservoir is almost full, a droplet of medium is formed at the inlet. Repeat this operation for all the chambers of the chip. Then, using a 200-µL pipette, aspirate from each reservoir the amount of medium that is necessary to have all the medium at the inlet entering the chamber (it enters by capillarity). **e**, Unlike during medium change, during transfection it is extremely important that the transfection solution go completely inside the channel immediately to avoid inhomogeneities of cell transfection and toxicity. Thus, the reservoir should be emptied first, almost completely, and the transfection solution should then be injected near the inlet. Letting the solution enter the channel via capillarity guarantees an approximately constant flow rate during successive medium changes and transfections. **f**, Images of two stages during medium change: delivery of 12 µL of medium to the inlet of the culture chamber (left), and medium removal at the outlet/reservoir (right). Note that during medium removal the microfluidic chip is rotated by 180°. The two insets highlight the inlets of the channel after medium delivery and after medium entrance into the channel by capillarity. See also Supplementary Video 1. **g**, Time course of fibroblast morphology during proliferation within a microfluidic chamber at days (D) 1, 2, 4, 6, and 8 after seeding at 60 cell/mm^2.

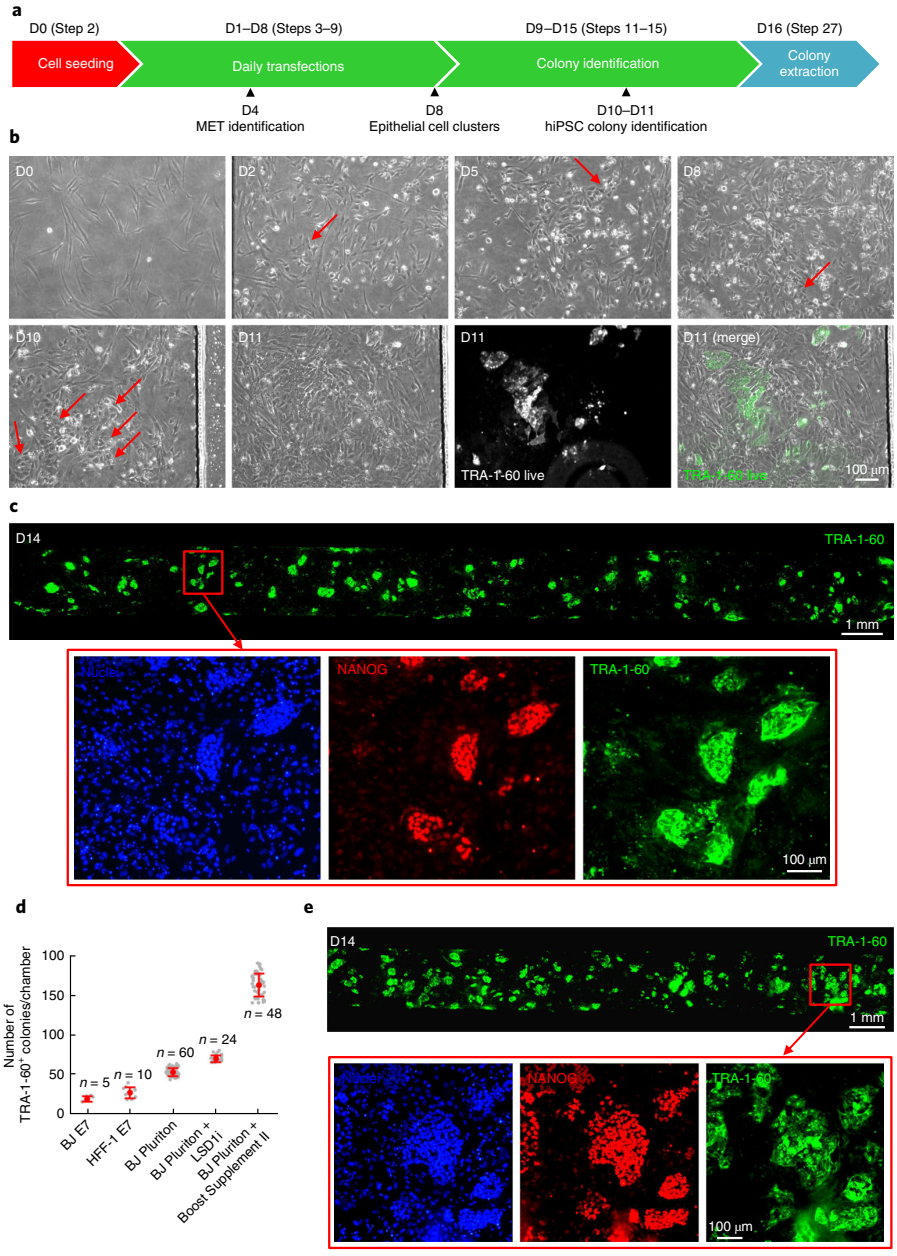

Fig. 4 | Expected results during the human fibroblast reprogramming process. a, Diagram of the workflow with the indicated critical morphological changes. **b**, Time-lapse images of cell morphology along the process. Red arrows highlight morphological hallmarks: epithelial morphology (D2), cell clusters at different compacting stages (D5 and D8), emerging hiPSC colonies (D10). TRA-1-60 live staining confirms the expression of this marker by newly formed hiPSC colonies by fluorescence microscopy (D11; scale bar, 100 µm). **c**, Top, representative image of immunofluorescence analysis of a whole culture chamber after 14 d of fibroblast reprogramming. We created the figure by tiling single microscopy images next to each other (scale bar, 1 mm). Bottom, enlargement of the region highlighted by the red frame in the whole-chamber image, assayed for Hoechst (blue), NANOG (red) and TRA-1-60 (green; scale bar, 100 µm). **d**, Dot plot indicating the number of hiPSC colonies obtained from each independent microfluidic culture chamber from the indicated fibroblast cell lines and using the indicated supplements. The superimposed red bars represent mean ± s.d. of the data. The number of replicates, *n*, is also indicated for each condition. **e**, Top, representative image of immunofluorescence analysis of TRA-1-60 in a culture chamber in which fibroblasts were reprogrammed in the presence of Human iPS Reprogramming Boost Supplement II for 14 d (scale bar, 1 mm). Bottom, enlargement of the region highlighted by the red frame in the top image, assayed for Hoechst (blue), NANOG (red) and TRA-1-60 (green; scale bar, 100 µm).

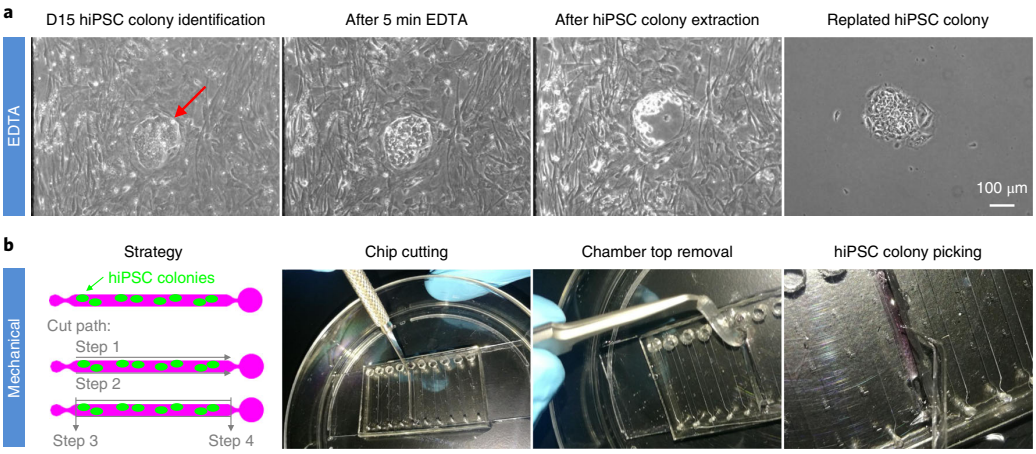

Fig. 5 | Methods of hiPSC colony extraction from the microfluidic culture system. a, Isolation of hiPSC colonies from a single culture chamber as a pool by EDTA treatment, and replating in a standard dish (scale bar, 100 µm). **b**, Procedure for mechanical picking of a single hiPSC colony from a chamber of the microfluidic device. See also Supplementary Video 2.

Box 1 | Microfabrication ● Timing ~9 h

The first three substeps shown in the workflow in Fig. 2 refer to the stages required to fabricate the final microfluidic setup used in reprogramming experiments. The techniques involved are well established in the field, and we refer the reader to a published protocol for details on replica molding (REM) for fabrication of microfluidic devices in PDMS[19]. We discuss how to adapt the protocol to the specific device used in this work. Reagents and equipment are listed in the Materials section of the main Procedure.

Procedure

1 Photomask design (Step 1 in ref. [19]). This step is necessary only if specific modifications to the layout proposed here are needed. The photomask for this protocol is provided as a high-resolution Supplementary Manual. The layout is described in Supplementary Fig. 2.

2 Master mold fabrication (Steps 4 and 6 in ref. [19]). This step requires photolithography to fabricate a master with a 200-µm-high pattern, using negative SU-8 2100 resist. This mold can be used several times.
▲ **CRITICAL STEP** Repeat Step 6 in ref. [19] every 10–15 uses of the mold for PDMS device production, to preserve it for long-term use.

3 To produce the microfluidic device (Steps 7–11 in ref. [19]), use Sylgard 184 PDMS in a 10:1 base:curing-agent ratio in order to obtain PDMS stamps of ~3-mm thickness.

4 Use disposable 1- and 3-mm biopsy punches to produce each culture chamber inlet and outlet hole, respectively, as shown in Fig. 1b.

5 Thoroughly clean a microscopy glass slide with MICRO-90 cleaning solution (2% (vol/vol) in distilled water) and rinse with distilled water. Alternatively, a rectangular glass coverslip can be used; in this case, cleaning is not needed.

6 Attach the patterned PDMS to a dry, clean glass slide, using an oxygen plasma cleaner (Steps 34–36 in ref. [19]).

7 Cool the bonded microfluidic device to room temperature (25 °C).

8 Pipette 20 µL of isopropanol into each culture chamber to clean the chamber of production process by-products, and rinse by flowing 30 µL of distilled water into the chambers before isopropanol evaporation.

9 Dry the chambers by aspirating the water with a 200-µL pipette and package the microfluidic device inside an autoclave bag.

10 Autoclave at 121 °C for 15 min, and let dry afterward.
■ **PAUSE POINT** The microfluidic devices can be kept in this state, at room temperature, indefinitely before use.

Materials

Biological materials

- Human fibroblasts (we have successfully used BJ cells (ATCC, cat. no. CRL-2522) and HFF-1 cells (ATCC, cat. no. SCRC-1041)) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.

Box 2 | Fibroblast culture in microfluidics ● Timing 3–4 h

Before using the microfluidic setup for cell reprogramming, it is useful to first test that the system allows fibroblast expansion. This test has two purposes: (i) to allow the user to become familiar with cell seeding and liquid handling in the microfluidic device, and (ii) to confirm that there are no problems with the device. Reagents and equipment are listed in the Materials section of the main Procedure.

Procedure

1 Prepare a vitronectin solution of 25 μg/mL in DPBS under sterile conditions and warm it to room temperature. Prepare 12 μL of solution per culture chamber, with an additional 10% excess volume. Alternatively, for fibroblast culture only, a fibronectin solution of 25 μg/mL in DPBS can be used.
▲ **CRITICAL STEP** For long-term culture, the quality of the glass surface coating is critical, and high-quality recombinant proteins, such as vitronectin, reduce experimental variability.

2 Under a laminar flow hood, extract a ten-chamber microfluidic chip from its sterile packaging, using sterile tweezers, and place it inside a 100-mm-diameter Petri dish.

3 Pipette 12 μL of vitronectin solution into each (empty) chamber, ensuring that the pipette tip is properly placed inside the 1-mm-diameter inlet before injection (Fig. 3b).

4 Add 1.5 mL of DPBS, optionally supplemented with penicillin–streptomycin, to the dish, around the microfluidic chip.

5 Incubate for 1 h at room temperature.

6 Pipette 12 μL of DMEM with 10% (vol/vol) FBS (or other fibroblast medium), optionally supplemented with penicillin–streptomycin, into each chamber, ensuring that the pipette tip is properly placed inside the 1-mm-diameter inlet before injection (Fig. 3b).

7 Aspirate the mixture of vitronectin solution and medium from the reservoir, avoiding complete emptying (Fig. 3b). Make sure there are no bubbles at the inlet after this procedure.
■ **PAUSE POINT** The chip can be left under the hood in this state for ~1 h before cell seeding. Place it at 4 °C for longer periods of time, preferably <~12 h. Bring it back to room temperature before cell seeding.

8 Prepare a cell suspension by detaching the fibroblasts from the previous culture dish and resuspending them in DMEM with 10% (vol/vol) FBS (or another fibroblast medium), according to standard biological practice. To seed cells at 60 cell/mm^2, a cell concentration in suspension of 300 cell/μL is required. Prepare 12 μL of solution per culture chamber, with an additional 10% excess volume.

9 Pipette 12 μL of well-mixed cell suspension into each chamber, ensuring that the pipette tip is properly placed inside the 1-mm-diameter inlet before injection. The injection should be performed quickly to guarantee spatial cell homogeneity along the microfluidic chamber (Fig. 3c).

10 Place the dish containing the seeded microfluidic chip in an incubator at 37 °C and 5% (vol/vol) CO_2.

11 After 24 h and approximately every 12 h after that, replace the medium in the microfluidic chambers with prewarmed fibroblast medium. For medium changes, deposit a 12-μL droplet at the inlet without pipetting inside the chamber; repeat the operation for each chamber of the chip; last, with a p200 pipette, aspirate the medium from each reservoir to let the medium enter the chambers by capillarity (Fig. 3d,f and Supplementary Video 1).
▲ **CRITICAL STEP** If medium changes every 12 h are not possible, avoid exceeding 15 h without a medium change.

12 Add 1 mL of sterile water, optionally supplemented with penicillin–streptomycin, to the dish, around the microfluidic chip, when the DPBS added at step 4 dries out.

13 Use phase-contrast microscopy to observe the fibroblast growth during culture for a few days (Fig. 3g).

Reagents

- Trypsin-EDTA (0.25% (wt/vol); Gibco, cat. no. 25200056)
- DMEM, high glucose (Gibco, cat. no. 11965)
- FBS; (Gibco, cat. no. 10099141)
- Vitronectin (VTN-N; Gibco, cat. no. A14700)
- Fibronectin (Corning, cat. no. CB-40008)
- Dulbecco's phosphate-buffered saline (DPBS; Gibco, cat. no. 14190144)
- Sterile distilled water
- Penicillin–streptomycin (Gibco, cat. no. 15140122)
- SU-8 2100 negative resist (MicroChem)
- SYLGARD 184 silicone elastomer kit (Dow Corning) ▲ **CRITICAL** The SYLGARD 184 silicone elastomer kit enables long-term cell culture; other elastomeric silicones are untested, and users should check to make sure they will work with the protocol.
- StemMACS mRNA transfection kit (Miltenyi, cat. no. 130-104-463)
- *eGFP* mRNA (optional) (Stemgent, cat. no. 05-0020) ▲ **CRITICAL** *eGFP* mRNA can be used during the protocol to visualize the transfection efficiency.
- StemRNA-NM reprogramming kit (Stemgent, cat. no. 00-0076) ▲ **CRITICAL** Conditions for using other commercially available RNA-NM reprogramming kits or custom-made RNA need to be optimized because they could have a strong effect on the reprogramming efficiency.
- RNaseZap RNase decontamination solution (Invitrogen, cat. no. AM9780)

- Pluriton medium (Stemgent, cat. no. 00-0070)
- Essential 6 medium (E6; Gibco, cat. no. A1516401)
- Fibroblast growth factor 2 (FGF2; Peprotech, cat. no. 100-18B-1000)
- StemMACS iPS-Brew XF, human (Miltenyi Biotec, cat. no. 130-104-368)
- TeSR-E8 kit for hESC/hiPSC cell maintenance (E8; Stem Cell Technologies, cat. no. 05990)
- ROCK Inhibitor StemMACS Y27632 (Miltenyi Biotec, cat. no. 130-103-922)
- LSD1 inhibitor, RN-1 (EMD Millipore, cat. no. 489479)
- Human iPS Reprogramming Boost Supplement II (EMD Millipore, cat. no. SCM094)
- StainAlive TRA-1-60 antibody (DyLight 488), mouse anti-human (Stemgent, cat. no. 09-0068)
- Pierce 16% (wt/vol) formaldehyde (methanol-free; Thermo Fisher Scientific, cat. no. 28906) **! CAUTION** Formaldehyde causes acute oral, dermal and inhalation toxicity. Handle it according to the manufacturer's instructions.
- Matrigel matrix, growth-factor reduced (GFR; Corning, cat. no. L003975)
- EDTA (0.5 M; pH 8.0; Thermo Fisher Scientific, cat. no. AM9260G)
- Horse serum (Thermo Fisher Scientific, cat. no. 16050122)
- Triton X-100 (Sigma, cat. no. T8787-100ML) **! CAUTION** Triton X-100 is harmful if swallowed, causes skin irritation and serious eye damage, and is very toxic to aquatic life, with long-lasting effects. Handle it according to the manufacturer's instructions.
- Bovine serum albumin (Sigma, cat. no. A3912-100G)
- Mouse anti-human OCT4 antibody (Santa Cruz, cat. no. sc-5279) 1:200 for immunofluorescence; 1:500 for western blotting
- Rabbit anti-human SOX2 antibody (Novus Biological, cat. no. NB110-37235) 1:200 for immunofluorescence; 1:500 for western blotting
- Rabbit anti-human NANOG antibody (Reprocell, cat. no. RCAB004P-F) 1:200 for immunofluorescence
- Rabbit anti-human NANOG antibody (Cell Signaling Techonolgy, cat. no. 4903) 1:1,000 for western blotting
- Mouse anti-human TRA-1-60 antibody (Millipore, cat. no. MAB4360) 1:200 for immunofluorescence
- Rabbit anti-human KLF4 polyclonal antibody (Santa Cruz, cat. no. sc-20691) 1:200 for immunofluorescence; 1:500 for western blotting
- Anti-GAPDH (Cell Signaling Technology, cat. no. D16H11) 1:2,000 for western blotting
- Anti-vimentin (Genetex, cat. no. 100619) 1:2,000 for western blotting
- Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. no. A-11001)
- Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific, cat. no. A-11005)
- Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific, cat. no. A-11012)
- Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. no. A-11008)
- Hoechst 33342 (Thermo Fisher Scientific, cat. no. H3570)

Equipment

- Glass microscopy slides (Menzel, cat. no. 10756991)
- Coverslips, 24 × 60 mm (Menzel, cat. no. 15747592)
- Laminar flow hood
- CO_2 water-jacketed incubator, set at 37 °C, 5% (vol/vol) CO_2, and 5% (vol/vol) O_2
- Thermostatic water bath set at 37 °C
- Phase contrast and epifluorescence microscope
- Stereomicroscope
- Sterile tweezers
- Sterile scalpel
- Disposable borosilicate glass Pasteur pipettes
- DNase- and RNase-free 0.2- and 1.5-mL sterile microcentrifuge tubes
- RNase-free sterile 15-mL conical tubes
- RNase-free sterile 50-mL conical tubes
- RNase-free sterile aerosol-barrier tips (10, 20, 200 and 1,000 μL)

Table 1 | Composition of transfection solutions for a ten-chamber microfluidic chip

Low dose		
Solution 1	**Solution 2**	**Solution 3**
RNA mix: 0.9 µL	TR: 0.3 µL	Medium: 110.7 µL
TB: 3.7 µL	TB: 4.4 µL	
Medium dose		
Solution 1	**Solution 2**	**Solution 3**
RNA mix: 1.6 µL	TR: 0.47 µL	Medium: 104.4 µL
TB: 6.3 µL	TB: 7.3 µL	
High dose		
Solution 1	**Solution 2**	**Solution 3**
RNA mix: 1.9 µL	TR: 0.6 µL	Medium: 101.3 µL
TB: 7.5 µL	TB: 8.8 µL	

TB, transfection buffer; TR, transfection reagent.

- Polystyrene tissue culture–treated six-well plates (BD Falcon, cat. no. 353046)
- Polystyrene tissue culture–treated 24-well plates (BD Falcon, cat. no. 353047)
- 100-mm-diameter Petri dish

Reagent setup

Preparation of the RNA mix for reprogramming

Thaw the three vials of NM-RNAs provided in the StemRNA-NM reprogramming kit (OSKMNL NM-RNA, EKB NM-RNA, and NM-miRNAs) and prepare the RNA mix according to the manufacturer's instructions. The daily RNA mix used for reprogramming a ten-chamber microfluidic chip ranges from 0.9 to 1.9 µL (Table 1). We suggest preparing 2-µL aliquots in sterile RNase-free microcentrifuge tubes, so that a fresh aliquot can be thawed each day. Store the aliquots at −80 °C for up to 3 months. During reprogramming, thaw the required vials on ice and do not refreeze. ▲ **CRITICAL** First, use RNase ZAP to clean all the surfaces of the laminar flow hood; use RNase-free sterile 0.2-mL microcentrifuge tubes and RNase-free sterile aerosol-barrier tips. Keep the RNA mix on ice at all times.

Quality control of main reagents

The quality control steps we describe here are optional and are useful for verifying the quality of the main reagents. They can be performed either before starting the actual reprogramming experiment or for troubleshooting an unsuccessful experiment. In the first check, start a reprogramming experiment but stop ~24 h after the fourth transfection. Verify robust expression of the encoded proteins by immunocytochemistry (Supplementary Fig. 3a) and western blotting (Supplementary Fig. 3b). Testing of all six transcription factors is rarely needed; the pluripotency-related transcription factors (OCT4, NANOG, SOX2) should be given priority. At this point, cells should have already down-regulated the fibroblast marker vimentin (VIM), which can also be assayed by western blotting (Supplementary Fig. 3b). As variations in transfectability may exist in different cell lines, mRNA encoding green fluorescent protein (GFP) can be added to the transfection solution, and its induced expression can be verified by fluorescence microscopy starting 24 h after the first transfection.

Procedure

Cell seeding and RNA transfections ● Timing ~9 d

1 Prepare the ten-chamber microfluidic devices according to Box 1.

2 Day 0, at 2 PM. Carry out Steps 1–10 of Box 2 to seed human fibroblasts to be reprogrammed in microfluidics, and place them in an incubator set to hypoxic conditions (37 °C, 5% (vol/vol) CO_2, 5% (vol/vol) O_2) overnight. The incubator used should be set to hypoxic conditions for the whole duration of the experiment.
▲ **CRITICAL STEP** In parallel, we recommend seeding a few channels with fibroblasts that will not be transfected, as a control for any morphological changes that occur during reprogramming.

3 Day 1, 9 AM (~16 h later). Replace the medium with 12 µL of supplemented Pluriton medium per chamber (Fig. 3d,f and Supplementary Video 1). Alternatively, E7 medium (E6 supplemented with FGF2 at 100 ng/mL) can be used in place of supplemented Pluriton medium. The medium can be supplemented with penicillin–streptomycin, but this is optional.
▲ CRITICAL STEP Cells should be put in reprogramming medium at least 9 h before transfection.
▲ CRITICAL STEP Cells should have reached ~50–60% confluence before the first transfection.
? TROUBLESHOOTING

4 Day 1, 6 PM (~9 h later). Prepare a low-dose transfection solution at room temperature (25 °C), using the StemMACS mRNA transfection kit, keeping the RNA on ice. Specifically, prepare separately solution 1 and solution 2 according to Table 1, and gently mix each of them. Combine solution 2 and solution 1, and mix by gently pipetting four times.
▲ CRITICAL STEP Use RNase-free tubes and tips.
▲ CRITICAL STEP Use a freshly thawed aliquot of RNA each day.

5 Incubate the mixture at room temperature for 20 min.

6 Add the transfection solution (mixture of solution 1 and solution 2) to solution 3 (supplemented Pluriton medium), prewarmed to room temperature, and gently mix. Alternatively, E7 medium can be used in place of supplemented Pluriton medium. The medium can be optionally supplemented with penicillin–streptomycin.

7 Take the cells out of the incubator, empty the reservoirs of the microfluidic device, and gently pipette 12 µL of transfection solution into each chamber (Fig. 3e).
▲ CRITICAL STEP Steps 6 and 7 should take <15 min. Consider preparing the transfection mix multiple times if a high number of chips are reprogrammed.

8 Incubate the cells overnight at 37 °C in the hypoxic incubator.

9 On days 2–8, repeat Steps 3–8 with the following RNA doses (detailed in Table 1): day 2, low dose; days 3–5, medium dose; and days 6–8, high dose. On days 1–8, medium can be supplemented with LSD1 inhibitor at a final concentration of 1 µM to further promote mesenchymal-to-epithelial transition. On days 5–8, medium can be supplemented with Human iPS Reprogramming Boost Supplement II (containing sodium butyrate, PS48 and TGF-β RI Kinase Inhibitor IV) to further enhance reprogramming efficiency.
? TROUBLESHOOTING

hiPSC colony identification and growth ● Timing ~7 d

10 On days 9–15, replace the medium in the morning (~9 AM) and at night (~7 PM) with 12 µL per chamber of hiPSC expansion medium (e.g., StemMACS iPSBREW XF or E8 medium).

11 When hiPSC colonies start to be visible (~days 10–14), verify the expression of the pluripotency marker TRA-1-60 by live staining. To do this, first prepare a solution of Stemgent StainAlive TRA-1-60 antibody at 5 µg/mL and inject 12 µL per chamber.

12 Incubate the cells for 30 min in the incubator.

13 Inject 12 µL of medium per chamber and incubate for 5 min.

14 Repeat Step 13 twice.

15 Observe the culture chambers by fluorescence microscopy (excitation/emission: 488/525) (typical results can be seen in Fig. 4b).

16 Continue culture as described in Step 10.

Immunostaining for pluripotency markers in microfluidics ● Timing ~2 d

▲ CRITICAL The overall workload for immunofluorescence assay in microfluidics is similar to that in standard wells.

17 After hiPSC colony identification, some chambers can be used for hiPSC immunofluorescence characterization. Use a 12-µL volume per chamber for each of the following steps. First wash the chambers once with DPBS, and then incubate the cells with 4% (wt/vol) formaldehyde for 10 min at room temperature.

18 Wash the chambers three times for 5 min each time with DPBS.

19 Incubate the cells with blocking solution (DPBS with 10% (vol/vol) horse serum; add 0.1% (vol/vol) Triton X-100 for intracellular targets) for 45 min at room temperature.

20 Prepare final-concentration primary antibody solutions, inject into the microfluidic chambers, and incubate overnight at 4 °C. We recommend using the primary antibodies listed in the Materials section

21 Wash three times for 5 min each time with DPBS.
22 Inject secondary antibody solution and incubate for 2 h at room temperature. The secondary antibodies listed in the Materials section are needed if using the primary antibodies recommended in Step 20.
23 Wash with DPBS for 5 min.
24 Incubate with 1 μg/mL Hoechst in DPBS for 10 min at room temperature.
25 Wash with DPBS.
26 Image the cells with an epifluorescence microscope.

hiPSC colony extraction

27 Extract colonies by EDTA (option A) or manual picking (option B).

(A) **hiPSC colony extraction by EDTA** ● Timing **~4 h**

(i) Prepare a six-well plate coated with 0.5% (vol/vol in DMEM) Matrigel Reduced Factor (MRF) and incubate it for 1 h at room temperature. Alternatively, plates can be coated with vitronectin (VTN-N) according to the manufacturer's instructions.
▲ CRITICAL STEP The MRF-coated plate can be prepared in advance (up to 2 weeks). In this case, prewarm at 37 °C for 30 min before use.
(ii) Supplement the hiPSC expansion medium with 1 μL/mL of ROCK Inhibitor (RI).
(iii) Aspirate the MRF coating from the plates, avoiding drying, and replace with the medium prepared in Step 27A(ii).
(iv) Wash the microfluidic chamber three times with 12 μL of calcium- and magnesium-free DPBS.
(v) Wash the microfluidic chamber five times with 12 μL of 0.5 mM EDTA.
(vi) Incubate for 5–10 min at 37 °C until the hiPSC colonies have a less compact morphology (Fig. 5a).
▲ CRITICAL STEP If hiPSC colonies are detached, proceed to Step 27A(vii); otherwise, incubate for an additional 5 min.
(vii) Remove excess solution from the reservoir.
(viii) Pipette in and out of the chamber 12 μL of the medium prepared in Step 27A(ii).
(ix) Transfer the 12 μL of medium from Step 27A(viii) to the MRF-coated well plate.
(x) Under the microscope, check for the successful transfer of the clone; if the hiPSC clone/clones are still present in the chamber, repeat Step 27A(vii,viii).
(xi) Completely dry the microfluidic chambers, transferring the cell suspension medium to the MRF-coated plate.
(xii) After 48 h, replace the medium in the well with hiPSC expansion medium without RI.
(xiii) Expand and characterize the hiPSC clones as previously described[9,20] or according to the manufacturer's instructions for the products used.

(B) **hiPSC single-colony extraction by mechanical picking** ● Timing **~1 d**

(i) Prepare some Pasteur pipettes with curved tips, using a Bunsen burner, and autoclave them.
(ii) Perform Step 27A(i–vii), using a 24-well plate.
(iii) Use a stereomicroscope to identify the hiPSC colony to pick.
(iv) In sterile conditions, under a stereomicroscope, use a scalpel to cut the microfluidic chamber as described in Fig. 5b and Supplementary Video 2.
▲ CRITICAL STEP During the first two cuts (Fig. 5b), make sure to cut slightly outside the chamber to avoid pushing the ceiling of the chamber against the hiPSC colony.
(v) Use sterile tweezers to remove the chamber ceiling (Fig. 5b).
(vi) Add some medium to the open microfluidic culture chamber to avoid cell drying and improve optical observation.
(vii) Under the stereomicroscope, use a glass Pasteur pipette prepared in Step 27B(i) to mechanically scratch the colony from the surface and transfer it to the plate prepared in Step 27B(ii).
(viii) Repeat Step 27B(iii–vii) for additional colonies.
▲ CRITICAL STEP Avoid prolonged exposure of the cells to room temperature. Place the microfluidic system back into the incubator for 30 min between operations in different chambers.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
Step 3	Before the first transfection, cells did not reach 50–60% confluence	Cells have reduced proliferative ability Cells did not attach well during seeding owing to low-quality coating or high mortality	Repeat cell seeding with higher cell density (70–80 cell/mm^2) Change medium on day 1 at ~9 AM and ~7 PM, and continue on day 2 from Step 3. This will reduce reprogramming efficiency, but hiPSC colonies can still be obtained
Step 9	Cell number is not increasing during the first days of transfection	Cells have reduced proliferative ability Cells are sensitive to transfection toxicity	Use low doses of RNA transfection reagents Consider seeding more cells (70–80 cell/mm^2) if reprogramming of this cell line is repeated
	Cells are almost confluent at day 3 (Supplementary Fig. 4a)	Cells have high proliferative ability	Increase RNA dose to a high dose Consider seeding fewer cells (40–50 cell/mm^2) if reprogramming of this cell line is repeated
	Cell morphology does not show mesenchymal-to-epithelial transition at day 5 (Supplementary Fig. 4b)	Low transfectability of cells	Verify expression of transcription factors by immunofluorescence assay Repeat the process with a higher dose of RNA
	Cells with epithelial morphology are not making clusters at day 8	Some cell lines take more time to reprogram	Consider increasing the number of transfections. Use BJ or HFF-1 morphology at day 8 as a reference for deciding when to stop the transfections

Timing

Step 1, preparation of microfluidic devices: ~4 h
Step 2, cell seeding: ~3 h
Step 3, medium change: ~30 min for one chip each morning on days 1–8
Steps 4–9, RNA transfections: ~1 h for one chip each night on days 1–8
Step 10, medium change: ~30 min two times per day on days 9–15
Steps 11–16, TRA-1-60 staining and fluorescence microscopy observation: ~4 h
Steps 17–26, immunostaining for pluripotency markers in microfluidics: ~2 d
Step 27A, hiPSC colony extraction by EDTA: ~4 h
Step 27B, hiPSC single-colony extraction by mechanical picking: ~1 d
Box 1, step 1, (optional) photomask design: ~2 h, with prior knowledge of the software used
Box 1, step 2, master mold fabrication: 5 h
Box 1, step 3, microfluidic device production: 3 h
Box 1, steps 4–10, microfluidic device assembly and sterilization: 1 h
Box 2, steps 1–10, cell-seeding protocol: 3 h
Box 2, step 11, medium changes for one chip: ~10–30 min, twice per day
Box 2, steps 12 and 13, quality control of fibroblast culture: 10–20 min

Anticipated results

It is advisable to first set up the process using, as a standard, low-passage human-foreskin-derived fibroblasts, such as BJ or HFF-1 cells. This protocol is effective in obtaining hiPSC colonies in every culture chamber. The timing of the morphological changes of cells during reprogramming, from acquisition of an epithelial morphology to compaction of hiPSC colonies, is shown in Fig. 4. By days 3 and 4, cells start to change morphology toward an epithelial-like state, which is acquired by day 7. Between days 8 and 10, small clusters of epithelial cells start to emerge. Defined cell nuclei are visible with evident nucleoli. We found that on day 14, BJ reprogramming efficiency was 52 ± 5 TRA-1-60$^+$ colonies per chamber (mean ± s.d., $n = 60$), as shown in Fig. 4c and quantified in Fig. 4d (also see the Source Data).

The reprogramming efficiency can be further increased by the addition of previously reported reprogramming boosters. As proof of concept, we report the use of LSD1 inhibitor[21] (efficiency = 70 ± 5 colonies per chamber, mean ± s.d., $n = 24$) or a cocktail of sodium butyrate[22], PS48[23], and TGF-β RI kinase inhibitor IV[24] (efficiency = 160 ± 20 colonies per chamber, mean ± s.d., $n = 48$) (Fig. 4d and Source Data).

Reprogramming in E6 supplemented with FGF2 (100 ng/mL), without the addition of other reprogramming boosters, is less efficient than reprogramming in Pluriton medium: BJ efficiency is 19 ± 4 TRA-1-60$^+$ colonies per chamber (mean ± s.d., $n = 5$), and HFF-1 efficiency is 27 ± 7 TRA-1-60$^+$ colonies per chamber (mean ± s.d., $n = 10$), both by day 14 (Fig. 4d and Source Data). For improved reprogramming efficiency, if a high proliferation rate is observed in E7 medium, cells can be seeded at lower density (30 cells/mm^2), whereas in the case that transfections induce high toxicity, they can be kept at a low dose until cells reach ~60% confluence, Typically, the transfection is switched to a medium dose at day 5 or 6 for BJ or HFF-1 fibroblasts.

From our experience, the reprogramming efficiency is very variable from cell line to cell line[2]. In the Troubleshooting section, we give some suggestions on how to handle this, by changing various factors such as cell density, RNA transfection dose, and number of transfections. Given the high throughput and low cost of microfluidics, for new primary lines we recommend testing multiple reprogramming conditions in parallel, according to the principles described in the Troubleshooting section, in one or two microfluidic devices. Following this strategy, ~30% of the microfluidic chambers contain up to five colonies, which is sufficient for derivation of the few stable hiPSC lines usually required for use. Despite some flexibility being required to identify the optimal conditions, we consistently found a gain in efficiency when performing the process in microfluidics compared with that achived with standard well plates.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files.

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Author contributions

O.G., C.L., and N.E. designed the study and wrote the manuscript. O.G. and C.L. optimized and performed the reprogramming experiments. W.Q. and S.G. produced the microfluidic devices. W.Q., E.B., and E.T. helped with reprogramming experiments. E.B., E.T., and A.U. helped with cell characterization. N.E. oversaw the project.

Competing interests

O.G., C.L., and N.E. are co-inventors on patent applications describing the reprogramming and differentiation processes in microfluidics: application nos. PD2013A000220, IT UA20162645 and 102016000039189, and PCT/IB2017/052167. O.G. and N.E. are cofounders of uSTEM Srl and Onyel Biotech Srl.

Additional information

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For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The size of the microfluidic chamber was determined by previously published data (Luni et al, Nat Meth 2016) as optimal for reprogramming efficiency and for protocol robustness.
Data exclusions	No data were excluded.
Replication	Number of replicates equal or higher than 5.
Randomization	Not applicable, this study is not comparing conditions, but describing a protocol.
Blinding	Not applicable, this study is not comparing conditions, but describing a protocol.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☒	☐ Unique biological materials
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Mouse anti-human OCT4 antibody (SantaCruz, cat. # sc-5279, 1:200 for immunofluorescence, 1:500 for western blot). Rabbit anti-human SOX2 antibody (Novus Biological, cat. # NB110- 37235, 1:200 for immunofluorescence, 1:500 for western blot). Rabbit anti-human NANOG antibody (Reprocell, cat. # RCAB004P- F, 1:200 for immunofluorescence). Rabbit anti-human NANOG antibody (Cell Signaling Techonolgy, cat. #4903, 1:1000 for western blot). Mouse anti-human TRA-1-60 antibody (Millipore, cat. # MAB4360, 1:200 for immunofluorescence). Rabbit anti-human KLF4 polyclonal antibody (Santa Cruz, cat. # sc-20691, 1:200 for immunofluorescence, 1:500 for western blot). Anti GADPH (D16H11, Cell Signaling Technology, 1:2000 for western blot). Anti Vimentin (100619, Genetex 1:2000 for western blot). Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. # A-11001). Goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific, cat. # A-11005). Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific, cat. # A-11012). Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, cat. # A-11008).
Validation	The antibodies used were previously tested in the literature. In this work, positive and negative controls were included in the study design. For immunofluorescence and Western blot of pluripotency markers, H9 cell line (WA09 from Wicell) was used as positive control, and BJ fibroblasts as negative control.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	BJ from ATCC, cat. # CRL-2522. HFF-1 from ATCC, cat. # SCRC-1041.
Authentication	The authentication of all the cell lines was provided from the supplier.
Mycoplasma contamination	All cell lines were periodically tested to exclude mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	Does not apply.