Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by defined factors. Here, we describe a protocol for imaging mitochondrial permeability transition pore (mPTP) opening in reprogramming of somatic cells using a confocal microscope. We also describe a method to sort high and low mPTP opening somatic cells by calcein fluorescence and reprogram these sorted cells to iPSCs. These protocols are also suitable for imaging mPTP opening and uncovering the mechanisms of mPTP function in other cell fate conversions.
Protocol

Protocols for analysis of mitochondrial permeability transition pore opening in mouse somatic cell reprogramming

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SUMMARY

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by defined factors. Here, we describe a protocol for imaging mitochondrial permeability transition pore (mPTP) opening in reprogramming of somatic cells using a confocal microscope. We also describe a method to sort high and low mPTP opening somatic cells by calcein fluorescence and reprogram these sorted cells to iPSCs. These protocols are also suitable for imaging mPTP opening and uncovering the mechanisms of mPTP function in other cell fate conversions. For complete details on the use and execution of this protocol, please refer to Ying et al. (2018).

BEFORE YOU BEGIN

Prepare medium

© Timing: [20–30 min]

1. Prepare fibroblast medium and mouse embryonic stem cells (mES) medium.

| Fibroblast medium | Final concentration | Amount |
|-------------------|---------------------|--------|
| Reagent           |                     |        |
| DMEM              | n/a                 | 500 mL |
| FBS (Industria Argentina, NTC-HK008) | 10% | 56 mL |
| GlutaMAX          | 1%                  | 5.6 mL |
| NEAA              | 1%                  | 5.6 mL |
| Total             | n/a                 | 567.2 mL |
Prepare buffer and solution

- **Timing:** [2–3 h]

2. Prepare Modified HBSS Buffer and Polyethylenimine (PEI) solution.

**Modified HBSS buffer**
Modified HBSS is purchased from ThermoFisher (Thermo Fisher Scientific, 14175095).

**Note:** Compare with HBSS, it has glucose without calcium, magnesium and phenol red.

**PEI solution (1 mg/mL)**

- Add 100 mg PEI (Polysciences, 23966) to 100 mL ddH₂O.
- Heat the solution to 65°C–70°C for 5 min.
- Add 1 M HCl to adjust pH to neutral (6.8–7.2).
- Sterilize the solution using Millipore’s 0.22 μm Steriflip filter.
- Prepare 100 × 1 mL aliquots and store them at –20°C until use. Avoid repeated freezing and thawing. After thawing, it can be stored at 4°C for 3–4 weeks.

**Note:** ddH₂O is 18.2 Ω MilliQ sterilized H₂O.

△ CRITICAL: The pH of solution needs to be adjust to neutral (6.8–7.2) or it will affect the transfection efficiency.

Prepare cells

- **Timing:** [5–7 days]

3. Prepare Plat-E cells, Mouse embryonic fibroblasts and reprogramming cells.
   - Plat-E cells were cultured in fibroblast medium and used to package the retroviruses.
   - Mouse embryonic fibroblasts (MEFs) were derived from 13.5-day mouse embryos carrying a transgenic Oct4 promoter driving GFP expression and cultured in fibroblast medium. Before MEFs successfully reprogram into induced pluripotent stem cells (iPSCs), the GFP will not express. In our reprogramming system the iPSCs appeared at least at day 15. In our analysis, the reprogrammed cells were all at early stage of reprogramming process and had no GFP expression. MEFs were used at passage 2.
c. Prepare reprogramming cells. After medium was removed, MEFs were infected with retroviruses. After 24 h the retroviruses were removed and added new retroviruses for another 24 h and the cells were cultured in mES medium after viral supernatants were removed. The day that viral supernatants were removed was defined as day 0. mPTP opening was detected by using the calcein release assay at days 0, 3, 5 and 8.

Prepare viruses

© Timing: [2–3 days]

4. Retroviruses plasmid transfection.

Retroviruses were packaged by transfection of Plat-E cells with pMXs retroviral vectors containing the coding sequences of mouse Sox2, Klf4, Oct4 and c-Myc (SKOM, Yamanaka’s factors). The plasmids vectors were transfected by using polyethyleneimine (PEI) -mediated transfection. This process needs 12-13 h.

a. The Plat-E cells were incubated at 40% density to 100-mm dish 24 h before transfection.

b. Before transfection, replace the fibroblast medium with 10 mL fresh medium.

c. Prepare DNA-PEI complex:

Plasmids vectors were diluted into Optimized Medium (Gibco, 31985-070), mixed well, incubated at around 25°C for 5 min. PEI was added into the Optimized Medium containing plasmids, mixed well, incubated at around 25°C for 8–15 min. The reagent volumes are shown in Table 1.

d. Drop the Optimized Medium with DNA: PEI complex to the cells, and shake the dish gently to make the complex distribute evenly.

△ CRITICAL: Adding DNA: PEI complex to the cells needs to be done slowly and drop by drop to make the complex distribute evenly. If the distribution of the complex is not even, the transfection efficiency will be seriously affected.

e. Replace fibroblast medium with 10 mL fresh medium at 12 h after plasmid transfection.

5. Retroviruses collection. This process needs two days.

a. Viral supernatants are collected at 48 h after plasmid transfection. Add fresh fibroblast medium and collect viral supernatants 24 h later. Mix these viral supernatants.

b. Filter the viral supernatants with 0.45 μm Steriflip filter.

◆ Pause point: Viral supernatants can be stored up to 7 days at 4°C.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Hoechst 33342       | Thermo Fisher | Cat# H3570 |
| Bovine Serum Albumin (BSA) | Sigma | Cat#B2064-50G |
| Modified HBSS buffer | Thermo Fisher | Cat#14175095 |
| Dulbecco’s Phosphate-buffered saline (DPBS) | Gibco | Cat#14190144 |

(Continued on next page)
Detecting the opening of mPTP in reprogramming cells

Prepare reprogramming cells

© Timing: [3 days]

1. Coat 35 mm dish

Glass-bottom dishes have poor adhesion for cells. Coating dishes with gelatin (EmbryoMax 0.1% Gelatin Solution) can enhance MEF cells adhesion. Coat 35 mm dish with 1 mL gelatin for at least 0.5 h at 37°C.

2. Split MEFs
Remove the gelatin and plate MEFs at 40,000 cells per dish. Gently put the dish into a 37°C, 5% CO₂ incubator for 24 h for cell attachment.

**CRITICAL:** MEFs are likely to aggregate. Cells should be evenly distributed to facilitate viral infection, which is important for reprogramming. MEFs are likely to aggregate in the gelatin-coated dish, so the cells should be diluted to an appropriate amount before plating. For 35 mm dish, appropriate number of cells was diluted to 1 mL culture medium and mixed well. Add the cells into dish and gently rock the dish to distribute the cells.

*Note:* The proliferation rate of MEFs varies among different batches of mouse. We need to prepare 160,000 MEFs to repeat this experiment with appropriate cell confluence. The 30%-40% confluence of the initial cells will be hard for us to image at day 8 because of the rapid emergence of clones (Figure 1). So we need to prepare appropriate cell number in each dish and don’t plate more than 40,000 cells per dish.

3. Infect MEFs with SKOM retroviruses

Remove the medium and add 2.5 mL SKOM viral supernatants to dish with 24 h incubation and infect two rounds (24 h each round).

**Calcein release assay**

**Timing:** [30–45 min]

This assay employs calcein-AM, a colorless and nonfluorescent esterase substrate, and CoCl₂, a quencher of calcein fluorescence, to selectively label mitochondria. Cells are loaded with the acetoxymethyl ester of calcein dye, calcein-AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleave the acetoxymethyl esters to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of CoCl₂, while the fluorescence from the mitochondrial calcein is maintained. When mPTP is open, more CoCl₂ will go into mitochondria and quench calcein. The calcein fluorescence intensity indicates the opening of mPTP.

4. Wash cells twice with modified HBSS buffer.

5. Incubate cells in 1 mL labeling solution for 15 min protected from light at 37°C.

*Note:* Labeling solution must be used immediately

6. After incubation, cells were washed with 1 mL warm modified HBSS buffer for once to remove residual dye and minimize background.

*Note:* Calcein release assay was carried out according to manufacturer’s instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp35103.pdf&title=SW1hZ2UtaVQgTEIWR5BNaXrvY2hvbmRyaWFsIFRyYW5zazXRpb24gUG9yZSB8c3NheSBLaXQ=) in Transition Pore Assay Kit. The hoechst and CoCl₂ were stock solution in the Kit, we need to prepare the stock solution of calcein-AM (1 mM) with DMSO. Prepare labeling solution with these stock solutions and label cells for imaging as described at step 5.
Confocal imaging

**Timing:** [2 h/day]

7. Image cells with Zeiss LSM710 (Calcein: Ex 488 and Emi 509; DAPI: Ex 405 and Emi 420) and find the reprogramming cells.
   a. At day 0, the reprogramming just begin and we can’t select which cell will undergo reprogramming, so we image the cells at random.
   b. The cells will go through MET (mesenchymal - epithelial transition) during reprogramming (Li et al., 2010) and we found the cells begin undergoing epithelial-like morphological change from elongated cells to rounded cells at day 3 in our system (Ying et al., 2016 and Figure 1A). So image the morphology changed cells which are reprogramming cells at days 3, 5 and 8 during reprogramming process as shown in Figure 1A.

Figure 1. Calcein release assay
(A) The morphological change occurs during reprogramming. The amount of cells is appropriate (Day 8-a) compared with too many ones, which form clones (Day 8-b). Scale bar corresponds to 100 μm.
(B) Opening of mPTP was analyzed at days 0, 3, 5, and 8 during reprogramming. Scale bar corresponds to 20 μm.
c. Prepare four dishes and the mPTP opening analysis was carried out at days 0, 3, 5 and 8 during reprogramming as shown in Figure 1B.

**Note:** In our system, the day that viral supernatants were removed was defined as day 0.

**Alternatives:** Most confocal microscopes can image mPTP opening, such as Zeiss LSM 880 or Leica DMIRE2 inverted microscope.

### Sorting high and low mPTP opening cells

**Culture MEFs**

**© Timing:** [2–3 days]

8. After quickly thawing MEFs in a 37°C water bath, immediately transfer the thawed cell into a 15 mL tube containing 5 mL of fibroblast medium.
9. Centrifuge the tube for 5 min at 200 g.
10. Discard the supernatant and transfer the cell to a 10 cm dish with 10 mL fibroblast medium and go to the next step at 2–3 days till the number of cells reach to 3–6 million (the confluence reaches to 60%–80%).

**Stain MEFs**

**© Timing:** [45–60 min]

11. Use 1 mL DPBS to wash cells once and add 1 mL 0.25% Trypsin-EDTA at 37°C for 2 min.
12. Add 1 mL fibroblast medium, suspend MEFs and centrifuge for 5 min at 200 g.
13. Suspend MEFs with modified HBSS buffer (Negative control, NC) or labeling solution without hoechst (modified HBSS buffer with 1 μM calcein and 1 mM CoCl₂) at a final concentration of 1 × 10⁶ cells/mL and incubate for 15 min protected from light at 37°C. The negative control is used to set up the MoFlo Astrios instrument with appropriate photomultiplier tubes (PMT) voltages refer to the guidelines for the use of flow cytometry (Cossarizza et al., 2019).
14. After incubation, labeled cells were washed with 1 mL modified HBSS buffer and centrifuged for 5 min at 200 g and washed for twice to remove residual dye and minimize fluorescent background.

**Sort the high and low mPTP opening cells**

**© Timing:** [2–4 h]

15. Resuspend MEFs to 2% BSA in DPBS buffer and sort the mPTP opening cells according to calcein fluorescence intensity. Before sorting, we need to prepare the tubes with medium to collect the sorted cells. We also need to prepare the gelatin-coated 12-well plate for cell attachment after the sorting.

△ **CRITICAL:** The 12-well plate must be coated with gelatin otherwise many cells will die.
16. Set the sorting parameters of flow cytometry with 488 nm excitation. We first gate the appropriate cells with signal cells and without debris (gate R1 and R2) and go to G2. And then in G2 we gate the low (R3) and high (R4) calcein fluorescence intensity cells (mean fluorescence intensity of high is about 10 times more than that of low) (Figure 2).

17. Sort the cells with low and high calcein fluorescence intensity indicating high and low mPTP opening, respectively.

Perform the reprogramming

© Timing: [18 days]

18. Plate high and low mPTP opening cells to 12-well plate and incubate for 12 h for cell attachment.

19. Infect the cells with SKOM retroviruses as described previous (In Prepare Viruses section and Prepare Cells section of Reprogramming cells)

20. Count the efficiency of reprogramming at day 15. The reprogramming efficiency was determined by counting GFP-positive iPSC colonies.

EXPECTED OUTCOMES

Mitochondrial permeability transition pore (mPTP) is permeable to any molecule of <1.5 kDa in size. Its prolonged opening results in cell death. Transient mPTP opening has physiological roles unrelated to death stimuli, such as Ca$^{2+}$ release and redox equilibrium (Rasola and Bernardi, 2007). Here we image the transient opening of mPTP during reprogramming which may play a physiological role in reprogramming. If we find the changes of mPTP status in reprogramming, it may indicate that mPTP has physiological function in the conversion of somatic cells into pluripotent ones.

Following this protocol, we developed a method for analysis of mPTP opening during reprogramming and another method of sorting the high and low mPTP opening cells for reprogramming. Too many the initial cells will result in formation of clones at day 8 during reprogramming (Figure 1A). With appropriate cells, the result showed that opening of the mPTP increased at day 3, then decreased at day 5 and maintained at that level at day 8 (Figure 1B). The MEFs could be sorted by low and high mPTP opening through flow cytometry with the calcein fluorescence intensity (Figure 2). In other cells fate conversion, these protocols are also suitable. With these methods we could analyze the status of mPTP at different stage of cells fate conversion and uncover the mechanisms of mPTP function.

LIMITATIONS

Our protocol is designed for reprogramming cells. In our system, we need to select the reprogramming cells and these cells are easy to form clones during the reprogramming, which need to be taken into consideration for mPTP opening analysis. We don’t think in other cell lines or primary cells will have these problems, and you don’t need to follow our protocol completely. The manufacturer’s instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2F manuals%2Fmp35103.pdf&title=SW1hZ2UtavaVQgTEIWR5BNaXRvY2hvbmRyaWFsIFRoYW5zaXRpb24gUG9yZSBc3Nhese5BLaXQ=) in Transition Pore Assay Kit is enough for analysis.

In our protocol, the MEFs were derived from 13.5-day mouse embryos carrying a transgenic Oct4 promoter driving GFP expression. If there is no access to this mice, Alkaline phosphatase (AP) staining of cells or FACS analysis of SSEA1-positive cells at day 15 of reprogramming are also the ways to indicate the efficiency of reprogramming. Alkaline phosphatase staining could be performed with NBT/BCIP (Roche, 11697471001). The cell surface marker SSEA1 can also be used to detect the reprogramming cells. Cells were incubated with anti-mouse SSEA1 PE (eBioscience, 50-8813-41) and
analyzed on a FACS Aria (BD Biosciences). These two methods can indicate the efficiency of reprogramming, however not all of AP and SSEA1 positive cells can be converted into induced pluripotent stem cells (iPSCs).

Hu and colleagues have used both above-mentioned methods to uncover certain mechanisms for reprogramming (Hu et al., 2014). However, it is important to note that Oct4-GFP fluorescence is easy, accurate and stable for detecting the efficiency of reprogramming.

TROUBLESHOOTING

Problem 1
Mesenchymal-to-epithelial transition doesn’t appear at day3 during reprogramming (step 7)

Potential solution
There are two reasons about this problem. 1) The infection efficiency of SKOM retroviruses is low and the reprogramming failed; 2) The dish is coated with too little gelatin or the coating time is not enough, which result in poor adhesion of cells. We need to improve the infection efficiency of retroviruses and increase the amount of gelatin and the coating time.

Problem 2
The calcein fluorescence intensity is low or hard to detect (steps 4–7 and 10–14)

Potential solution
Stock solution of calcein-AM (1 mM) may be expired. The stock solution should not be stored at –4°C for more than 2 weeks.
Problem 3
The calcein fluorescence diffuses in cells other than mitochondria or there is calcein fluorescence in cytosolic area (steps 4–7)

Potential solution
The CoCl₂ may not be enough to quench cytosolic fluorescence. We need to increase the amount of CoCl₂.

Problem 4
Cell aggregation occurs when cells were added into dish (step 2).

Potential solution
Add the cells directly into dish without dilution will cause cell aggregation. We need to dilute the cells to 1 mL medium, mix well, add the cells into the dish, and then shake them gently.

Problem 5
The sorted cells have low survival rates (steps 12–14)

Potential solution
Suspend MEFs to 2% FBS (Industria Argentina, NTC-HK008) instead of BSA in DPBS buffer before sorting the high and low mPTP opening cells.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xingguo Liu (liu_xingguo@gibh.ac.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze new datasets/code.

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AUTHOR CONTRIBUTIONS
X. L. conceived, designed, and supervised the project. Z. Y., Z. L., and Y. X. conducted most of the experiments and performed data analysis. J. W. participated in the flow cytometry. Z. Y., Z. L., X. G., and X. L. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES

Cossarizza, A., Chang, H.D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W.W., Aghaeepour, N., Akdis, M., Allez, M., et al. (2019). Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur. J. Immunol. 49, 1457–1973.

Li, R., Liang, J., Ni, S., Zhou, T., Qing, X., Li, H., He, W., Chen, J., Li, F., Zhuang, Q., et al. (2010). A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell 7, 51–63.

Hu, X., Zhang, L., Mao, S.Q., Li, Z., Chen, J., Zhang, R.R., Wu, H.P., Gao, J., Guo, F., Liu, W., et al. (2014). Tet and TDG mediate DNA demethylation essential for mesenchymal-to-epithelial transition in somatic cell reprogramming. Cell Stem Cell 14, 512–522.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.

Ying, Z., Chen, K., Zheng, L., Wu, Y., Li, L., Wang, R., Long, Q., Yang, L., Guo, J., Yao, D., et al. (2016). Transient activation of mitoflashes modulates nanog at the early phase of somatic cell reprogramming. Cell Metab. 23, 220–226.

Ying, Z., Xiang, G., Zheng, L., Tang, H., Duan, L., Lin, X., Zhao, Q., Chen, K., Wu, Y., Xing, G., et al. (2018). Short-term mitochondrial permeability transition pore opening modulates histone lysine methylation at the early phase of somatic cell reprogramming. Cell Metab. 28, 935–945.e5.

Rasola, A., and Bernardi, P. (2007). The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12, 815–833.