Mitochondrial dynamics play critical roles in both tissue homeostasis and somatic cell reprogramming. Here, we provide integrated guidance for assessing mitochondrial function and dynamics while reprogramming human fibroblasts via an integrated analysis approach. This protocol includes instructions for mitochondrial metabolic analysis in real time and flow cytometry-based assessment of mitochondrial mass and membrane potential. We also describe a protocol for quantification of mitochondrial network and key metabolites.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Integrative analysis of mitochondrial metabolic dynamics in reprogramming human fibroblast cells

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SUMMARY

Mitochondrial dynamics play critical roles in both tissue homeostasis and somatic cell reprogramming. Here, we provide integrated guidance for assessing mitochondrial function and dynamics while reprogramming human fibroblasts via an integrated analysis approach. This protocol includes instructions for mitochondrial metabolic analysis in real time and flow cytometry-based assessment of mitochondrial mass and membrane potential. We also describe a protocol for quantification of mitochondrial network and key metabolites. For complete details on the use and execution of this protocol, please refer to Cha et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for reprogramming human fibroblasts. In addition, as this protocol includes real-time metabolic flux assessment of live cells, both 5% CO2 and non-CO2 37°C incubators are required for performing the protocol operations. Furthermore, users need to have ready access to key equipment, including a Seahorse XF H5 Mini Analyzer, confocal microscope, and a flow cytometer (we used a MACSQuant® Analyzer 16 flow cytometer here, but flow cytometers from different providers also work with this protocol) to carry out these experiments.

Institutional permissions

The fibroblast cell lines used in this study are approved for research use by the National Institutes of Health. The study was approved by the Mass General Brigham Institutional Biosafety Committee (#2020B000269). Others who wish to replicate this protocol will need approval from their respective funding agencies and/or institutions.

Cell culture: Thawing and maintaining fibroblasts

© Timing: 3–4 days

The protocol below describes the steps for thawing and maintaining human fibroblasts (BJ and GM03529 obtained from ATCC and Coriell Institute, respectively).

Note: For users unfamiliar with primary culture of fibroblasts from skin biopsies, we recommend Kisiel and Klar’s protocol (Kisiel and Klar, 2019).

1. Thawing and maintaining fibroblasts.
   a. Pre-warm fibroblast medium in 37°C water bath for up to 30 min.
b. Bring the pre-warmed fibroblast medium into a laminar flow hood.
c. Add 7 mL of fibroblast medium into a 15 mL conical tube.
d. Bring a vial of frozen fibroblasts (1 × 10^6 cells/vial) from vapor-phase liquid nitrogen storage and rapidly thaw in 37°C water bath.
e. When only a small piece of ice particle is floating, sterilize the exterior of the vial with 70% ethanol and put it into the laminar flow hood.
f. Transfer the defrosted cells into the 15 mL conical tube containing 7 mL of fibroblast medium.

△ CRITICAL: Use care to avoid damaging cells that are fragile after thawing.

g. Spin the 15 mL conical tube at 200 × g for 5 min.
h. Carefully remove the supernatant and resuspend the cells in 5 mL of fibroblast medium.
i. Add the cell suspension to a T-25 flask and return it to the 5% CO₂ 37°C incubator.
j. Cells should be fed with 8 mL of pre-warmed fresh fibroblast media every other day until confluence approaches 80%–90%.

Human iPSC generation

© Timing: 2–3 weeks

The protocol below describes the steps for reprogramming human fibroblasts into embryonic stem cell-like state.

Alternatives: In this protocol, we used our episomal vector-based reprogramming method that combines conventional transcription factors (e.g., OCT4, SOX2, KLF4, and L-MYC) and metabolism-regulating microRNA clusters (e.g., miR-302s and miR-200c) (Song et al., 2020). However, any type of iPSC generation kits would be suitable for this protocol.

2. Preparing Matrigel-coated tissue culture plate.
   a. Thaw the Matrigel stock vial on ice for 5 h.

△ CRITICAL: Thaw the Matrigel stock solution completely. Do not hand warm the vial to prevent gelation.

b. Add 25 mL of cold DMEM/F12 medium into a 50 mL conical tube.
c. Add an appropriate amount of Matrigel stock solution to the 50 mL conical tube containing DMEM/F12 medium and vortex thoroughly.

△ CRITICAL: Dilution factor of Matrigel stock solution is lot-dependent. See the dilution factor provided in the manufacturer’s certificate of analysis.

d. Add 1 mL of diluted Matrigel solution into each well of 6-well plate and be sure to cover the entire surface of plate.
e. Leave the plate at room temperature (15°C–30°C) for at least 1 h.

3. Reprogramming of human fibroblasts.
   a. Pre-warm fibroblast medium in a 37°C water bath.
   b. Take out the flask containing fibroblasts from the 5% CO₂ 37°C incubator.
   c. Aspirate the medium and rinse the cells with 5 mL of DPBS twice.
   d. Aspirate the DPBS, add 5 mL of TrypLE and evenly distribute over the cells.
   e. Return the flask to the 5% CO₂ 37°C incubator.
   f. After incubation for 5 min, check cells under the microscope. If cells are still attached, return them to the incubator for up to 2 more min or until the cells start to detach.
g. Transfer the cell suspension to a 15 mL conical tube.

h. Add 9 mL of fibroblast medium to the plate and collect any remaining cells to the tube.

i. Spin the tube at 200 × g for 5 min.

j. Carefully remove the supernatant and resuspend the cells in 5 mL of DPBS.

k. Remove a 10 μL aliquot of cell suspension for cell counting.

**Note:** Check the cell concentration and viability using trypan blue solution.

l. Spin the tube at 200 × g for 5 min.

m. Carefully remove the supernatant and resuspend the cells in Resuspension Buffer R (provided with the Neon™ Transfection System 100 μL Kit) to a concentration of 1 × 10^7 cells/mL.

n. Transfer 100 μL of cell suspension to a sterile 1.5 mL microcentrifuge tube.

o. Turn on the Neon unit and enter the following electroporation parameters in the Input window:

i. Pulse voltage: 1,650 V.

ii. Pulse width: 10 ms.

iii. Pulse number: 3.

p. Fill the Neon tube with 3 mL of Electrolytic Buffer E2 (provided with the Neon™ Transfection System 100 μL Kit) and insert the Neon tube into the Neon Pipette Station until you hear a click.

q. Transfer 9 μL of Episomal vector cocktail per transfection reaction to the tube containing the cells and mix gently.

r. Press the push-button on the Neon Pipette to the first stop and immerse the Neon tip into the cell-DNA mixture.

s. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon Tip.

t. Insert the Neon Pipette with the sample vertically into the Neon Tube placed in the Neon Pipette Station until you hear a click.

u. Press "Start" on the Neon touchscreen to deliver the electric pulse.

v. Remove the Neon Pipette from the Neon Pipette Station and immediately transfer the samples from the Neon Tip into the 15 mL conical tube containing 5 mL of pre-warmed plating medium.

w. Gently mix the transfected cells and add 1 mL of cell suspension into the Matrigel-coated 6 well plate containing 1 mL of plating medium.

⚠️ **CRITICAL:** Use care to avoid damaging cells that are fragile after electroporation.

x. Evenly distribute cells across the plate and return it to the 5% CO₂ 37°C incubator.

y. From next day on, feed cells with 3 mL of NutriStem® hiPSC XF medium every day until ESC-like colonies are formed.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-TOM20 (used at 1:100 dilution) | Santa Cruz Biotechnology | Cat# sc-17764; RRID: AB_628381 |
| Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (used at 1:1,000 dilution) | Jackson Immunoresearch Laboratories | Cat# 715-546-151; RRID: AB_2340850 |
| Chemicals, peptides, and recombinant proteins | Agilent Technologies | Cat# 103059-100 |
| Seahorse XF calibrant solution | Agilent Technologies | Cat# 103335-100 |
| Seahorse XF base medium, without phenol red | Agilent Technologies | Cat# 103335-100 |

(Continued on next page)
## Protocol

### Critical commercial assays

| Assay                          | Source                        | Identifier   |
|-------------------------------|-------------------------------|--------------|
| Seahorse XFp Cell Mito Stress Test Kit | Agilent Technologies | Cat# 103010-100 |
| Citrate Colorimetric Assay Kit | BioVision                     | Cat# K655-100 |
| Alpha-Ketoglutarate Colorimetric Assay Kit | BioVision | Cat# K677-100 |
| Malate Colorimetric Assay Kit  | BioVision                     | Cat# K637-100 |
| Oxalacetate Colorimetric Assay Kit | BioVision | Cat# K659-100 |
| Neon Transfection System 100 μL Kit | Thermo Fisher Scientific | Cat# MPK10096 |
| JC-1 Dye                       | Thermo Fisher Scientific      | Cat# T316-8 |
| MitoTracker™ Green FM          | Thermo Fisher Scientific      | Cat# M7514    |

### Experimental models: Cell lines

| Cell line                        | Source                        | RRID          |
|----------------------------------|-------------------------------|---------------|
| Human BJ newborn dermal fibroblasts | ATCC                          | CRL-2522; CVCL_3653 |
| Human adult dermal fibroblasts    | Coriell Institute             | GM03529; CVCL_7394 |

### Recombinant DNA

| DNA                              | Source                        | Identifier   |
|----------------------------------|-------------------------------|--------------|
| pCXLE-OKSiM                      | (Song et al., 2020)           | N/A          |
| pCXLE-miR-3052a/200c             | (Song et al., 2020)           | N/A          |
| pCXWB-EBNA1                      | Addgene                       | Cat# 37624   |

### Software and algorithms

| Software                          | Source                        | URL                        |
|-----------------------------------|-------------------------------|-----------------------------|
| Seahorse WAVE Desktop Software    | Agilent Technologies          | https://www.agilent.com     |
| FlowJo                            | Becton Dickinson & Company    | https://www.flowjo.com      |
| GraphPad Prism 8                   | GraphPad Software             | https://www.graphpad.com    |
| Fiji                              | ImageJ.net                    | https://imagej.net          |
| MiNA (Valente et al., 2017)       | (Valente et al., 2017)        | https://github.com/StuartLab/MiNA |

### Other

| Equipment                        | Source                        | Identifier   |
|----------------------------------|-------------------------------|--------------|
| Seahorse XF HS Mini Analyzer     | Agilent Technologies          | Cat# 103022-100 |
| Seahorse XFp FluxPak             | Agilent Technologies          | Cat# T316-8   |
| BD Luer-Lok™ 50 mL syringe       | Becton Dickinson & Company    | Cat# 309653   |
| Synergy HTX multi-mode reader     | BioTek Instruments            |               |

(Continued on next page)
Filter the medium with a 0.22 μm filter unit.

Make several 50 mL aliquots in conical tubes to be used throughout the culture process and label each with initial, date and time of preparation and expiration date.

The medium can be stored in the dark at 4°C for up to 4 weeks.

Aliquot 9 mL of episomal vector cocktail in sterile 1.5 mL microcentrifuge tubes and store at −20°C for up to 6 months.

### MATERIALS AND EQUIPMENT

#### Fibroblast medium

| Reagents                  | Final concentration | Amount  |
|---------------------------|---------------------|---------|
| DMEM                      | 1x                  | 419.5 mL|
| FBS                       | 15%                 | 75 mL   |
| NEAA (100x solution)      | 1x                  | 5 mL    |
| β-ME (55 mM solution)     | 55 μM               | 500 μL  |
| Total                     | n/a                 | 500 mL  |

- Filter the medium with a 0.22 μm filter unit.
- Make several 50 mL aliquots in conical tubes to be used throughout the culture process and label each with initial, date and time of preparation and expiration date.
- The medium can be stored in the dark at 4°C for up to 4 weeks.

### Episomal vector cocktail

| Vectors                  | Stock concentration | Amount  |
|--------------------------|---------------------|---------|
| pCXLE-OSKlM              | 1 μg/μL             | 60 μL   |
| pCXLE-miR-302s/200c      | 1 μg/μL             | 20 μL   |
| pCW8-EBNA1               | 1 μg/μL             | 10 μL   |
| Total                    | n/a                 | 90 μL   |

- Aliquot 9 μL of episomal vector cocktail in sterile 1.5 mL microcentrifuge tubes and store at −20°C for up to 6 months.
Filter the medium with a 0.22 μm filter unit.

- Plating medium should be freshly prepared for every new experiment.

Warm up the XF assay medium in 37°C water bath at atmospheric CO₂ conditions before use.

Reconstituted compounds should be used on the same day.

- XF assay medium should be freshly prepared in the laminar flow hood for every new experiment.

Reagents Final concentration Amount
---
**XF basal medium (w/o phenol red, pH 7.4)** 1x 4.68 mL
D-glucose (2.5 M solution) 10 mM 20 μL
Sodium pyruvate (100 mM solution) 5 mM 250 μL
GlutaMAX (100x solution) 1x 50 μL
**Total** n/a 5 mL

- Stock solutions for real-time metabolic analysis

Reagents Stock solution concentration Preparation procedures
---
Oligomycin 50 μM Add 252 μL of XF assay medium to stock vial and vortex thoroughly.
FCCP 50 μM Add 288 μL of XF assay medium to stock vial and vortex thoroughly.
Rotenone/antimycin A 25 μM Add 216 μL of XF assay medium to stock vial and vortex thoroughly.

- Dilution of stock solutions for real-time metabolic analysis

| Reagents                  | Final concentration | Preparation procedures                                                                 |
|---------------------------|---------------------|----------------------------------------------------------------------------------------|
| Oligomycin (50 μM solution) | 1 μM                | Mix 60 μL of stock solution with 240 μL of XF assay medium and vortex thoroughly.       |
| FCCP (50 μM solution)     | 0.125 μM            | To make 0.125 μM diluted solution, mix 7.5 μL of stock solution with 292.5 μL of XF assay medium and vortex thoroughly. |
|                           | 0.25 μM             | To make 0.25 μM diluted solution, mix 15 μL of stock solution with 285 μL of XF assay medium and vortex thoroughly. |
|                           | 0.5 μM              | To make 0.5 μM diluted solution, mix 30 μL of stock solution with 270 μL of XF assay medium and vortex thoroughly. |
|                           | 1 μM                | To make 1 μM diluted solution, mix 60 μL of stock solution with 240 μL of XF assay medium and vortex thoroughly. |
|                           | 2 μM                | To make 2 μM diluted solution, mix 120 μL of stock solution with 180 μL of XF assay medium and vortex thoroughly. |
| Rotenone/antimycin A      | 0.5 μM              | Mix 60 μL of stock solution with 240 μL of XF assay medium and vortex thoroughly.       |

Plating medium

| Reagents                  | Final concentration | Amount |
|---------------------------|---------------------|--------|
| DMEM                      | 1x                  | 41.95 mL |
| FBS                       | 15%                 | 7.5 mL  |
| NEAA (100x solution)      | 1x                  | 500 μL  |
| Y-27632                   | 10 μM               | 50 μL   |
| **Total**                 | n/a                 | 50 mL   |
• Diluted stock solutions should be used on the same day.

**Blocking solution for quantification of mitochondrial network**

| Reagents                      | Final concentration | Amount     |
|-------------------------------|---------------------|------------|
| DPBS (w/o Ca\(^{2+}\) and Mg\(^{2+}\)) | 1×                  | 49.45 mL   |
| Triton X-100                  | 0.1%                | 50 μL      |
| Horse serum                   | 1%                  | 500 μL     |
| Total                         | n/a                 | 50 mL      |

• Blocking solution should be freshly prepared for every new experiment and stored at 4°C until use.

**Washing solution for quantification of mitochondrial network**

| Reagents                      | Final concentration | Amount     |
|-------------------------------|---------------------|------------|
| DPBS (w/o Ca\(^{2+}\) and Mg\(^{2+}\)) | 1×                  | 499.5 mL   |
| Triton X-100                  | 0.1%                | 500 μL     |
| Total                         | n/a                 | 500 mL     |

• Washing solution can be stored at room temperature (15°C–30°C) for up to 1 month.

**Stock solution for assessment of mitochondrial mass**

| Reagents                      | Final concentration | Amount     |
|-------------------------------|---------------------|------------|
| DMSO                           | 1×                  | 496.12 μL  |
| MitoTracker\textsuperscript{®} Green FM | 150 μM                | 50 μg      |
| Total                         | n/a                 | 496.12 μL  |

• Store the reconstituted stock solution at −20°C for up to 6 months.

**Stock solution for assessment of mitochondrial membrane potential**

| Reagents | Final concentration | Amount |
|----------|---------------------|--------|
| DMSO     | 1×                  | 1 mL   |
| JC-1     | 5 mg/mL             | 5 mg   |
| Total    | n/a                 | 1 mL   |

• Store the reconstituted stock solution at −20°C for up to 6 months.

**Stock solutions for mitochondrial metabolite assay**

| Reagents                      | Stock solution concentration | Preparation procedures |
|-------------------------------|-----------------------------|------------------------|
| Citrate developer            | 1×                          | Add 220 μL of citrate assay buffer to stock vial and vortex thoroughly. |
| Citrate enzyme mix           | 1×                          | Add 220 μL of citrate assay buffer to stock vial and vortex thoroughly. |
| Citrate standard             | 100 nmol/μL                 | Add 100 μL of distilled water to stock vial and vortex thoroughly. |
| Diluted citrate standard     | 1 nmol/μL                   | Mix 10 μL of citrate standard solution with 990 μL of distilled water and vortex thoroughly. Freshly prepare for every new experiment. |
| α-ketoglutarate (α-KG)       | 1×                          | Add 220 μL of α-KG buffer to stock vial and vortex thoroughly. |
| α-KG development enzyme mix  | 1×                          | Add 220 μL of α-KG assay buffer to stock vial and vortex thoroughly. |
| α-KG standard                | 100 nmol/μL                 | Add 100 μL of distilled water to stock vial and vortex thoroughly. |

(Continued on next page)
● Store stock solutions for mitochondrial metabolite assay at –20°C for up to 2 months.

| Reagents                              | Final concentration | Preparation procedures                                                                 |
|---------------------------------------|---------------------|---------------------------------------------------------------------------------------|
| Diluted α-KG standard solution        | 1 nmol/μL           | Mix 10 μL of α-KG standard solution with 990 μL of distilled water and vortex thoroughly. Freshly prepare for every new experiment. |
| Malate enzyme mix                     | 1x                  | Add 220 μL of malate buffer to stock vial and vortex thoroughly.                        |
| WST substrate                         | 1x                  | Add 1.05 mL of distilled water to stock vial and vortex thoroughly.                    |
| Malate standard                       | 100 nmol/μL         | Add 100 μL of distilled water to stock vial and vortex thoroughly.                     |
| Diluted malate standard solution      | 1 nmol/μL           | Mix 10 μL of malate standard solution with 990 μL of distilled water and vortex thoroughly. Freshly prepare for every new experiment. |
| Oxaloacetate (OAA) enzyme mix         | 1x                  | Add 220 μL of OAA buffer to stock vial and vortex thoroughly.                          |
| OAA developer                         | 1x                  | Add 220 μL of OAA buffer to stock vial and vortex thoroughly.                          |
| OAA standard                          | 100 nmol/μL         | Add 100 μL of distilled water to stock vial and vortex thoroughly.                     |
| Diluted OAA standard solution         | 1 nmol/μL           | Mix 10 μL of OAA standard solution with 990 μL of distilled water and vortex thoroughly. Freshly prepare for every new experiment. |

● 1× Reaction Mix solution for citrate assay should be freshly prepared for every new experiment and warmed up at room temperature (15°C–30°C) before use.

| Reagents                              | Final concentration | Amount per sample well | Amount per standard well |
|---------------------------------------|---------------------|------------------------|--------------------------|
| Citrate assay buffer                  | 1x                  | 44 μL                  | 46 μL                    |
| Citrate enzyme mix                    | 1x                  | 2 μL                   | –                        |
| Citrate developer                     | 1x                  | 2 μL                   | 2 μL                     |
| Citrate probe                         | 1x                  | 2 μL                   | 2 μL                     |
| Total                                 | n/a                 | 50 μL                  | 50 μL                    |

● 1× Reaction Mix solution for α-KG assay should be freshly prepared for every new experiment and warmed up at room temperature (15°C–30°C) before use.

| Reagents                              | Final concentration | Amount per sample well | Amount per standard well |
|---------------------------------------|---------------------|------------------------|--------------------------|
| α-KG assay buffer                     | 1x                  | 44 μL                  | 46 μL                    |
| α-KG converting enzyme mix            | 1x                  | 2 μL                   | –                        |
| α-KG development enzyme mix           | 1x                  | 2 μL                   | 2 μL                     |
| α-KG probe                            | 1x                  | 2 μL                   | 2 μL                     |
| Total                                 | n/a                 | 50 μL                  | 50 μL                    |

● 1× Reaction Mix solution for malate assay should be freshly prepared for every new experiment and warmed up at room temperature (15°C–30°C) before use.

| Reagents                              | Final concentration | Amount per well |
|---------------------------------------|---------------------|-----------------|
| Malate assay buffer                   | 1x                  | 38 μL           |
| Malate enzyme mix                     | 1x                  | 2 μL            |
| WST substrate                         | 1x                  | 10 μL           |
| Total                                 | n/a                 | 50 μL           |
1 × Reaction Mix solution for OAA assay

| Reagents           | Final concentration | Amount per sample well | Amount per standard well |
|--------------------|---------------------|------------------------|--------------------------|
| OAA assay buffer    | 1 x                 | 44 μL                  | 46 μL                    |
| OAA enzyme mix      | 1 x                 | 2 μL                   | –                        |
| OAA developer       | 1 x                 | 2 μL                   | 2 μL                     |
| OAA probe           | 1 x                 | 2 μL                   | 2 μL                     |
| Total               | n/a                | 50 μL                  | 50 μL                    |

- 1 × Reaction Mix solution for OAA assay should be freshly prepared for every new experiment and warmed up at room temperature (15°C–30°C) before use.

**STEP-BY-STEP METHOD DETAILS**

**Real-time metabolic analysis of human fibroblasts**

© Timing: 2 days

Below we provide a detailed step-by-step protocol about the real-time metabolic analysis of reprogramming human fibroblasts by using Seahorse XF HS Mini Analyzer.

**Alternatives:** This protocol includes the use of a commercial kit (Seahorse XFp Cell Mito Stress Test Kit from Agilent Technologies). However, users can perform the experiment using in-house made stock solutions (Zhang et al., 2012).

1. Plating fibroblasts onto an XF HS miniplate.
   a. Seed human fibroblasts onto wells B-G of the XF HS miniplate containing 100 μL of fibroblast medium at 5–6 × 10³ cells/well (~90% confluency).

   △ CRITICAL: When seeding, make sure to uniformly disperse the cells at the bottom of the well. Do not seed the cells on wells A and H of the miniplate as these are for blank/background correction.

   b. Incubate the XF HS miniplate in the 5% CO₂ 37°C incubator for 18–24 h.

2. Hydrating sensor cartridge.
   a. Put cartridges into the laminar flow hood.
   b. Separate the sensor cartridge from the utility plate and place the sensor cartridge upside down.
   c. Add 200 μL of XF Calibrant solution into each well of the Utility plate.
   d. Add 400 μL of XF Calibrant solution into the moat chambers around the outside of the wells.
   e. Return the XF Sensor cartridge to the Utility plate containing XF Calibrant solution.
   f. Place the cartridge assembly in a non-CO₂ 37°C incubator for 18–24 h.

   △ CRITICAL: The non-CO₂ 37°C incubator should be kept humidified at 100% to prevent evaporation of the XF Calibrant solution.

3. Preparing XF HS miniplate for assay.
   a. Next day, verify the condition of the cells in the XF HS miniplate before starting the assay ensuring the cells are well distributed.

   **Note:** Make sure the cells are well-attached at 80%–90% density as single cells, not as aggregates.

   b. Turn on the XF HS Mini Analyzer at least 1 h before the experiment.
c. Prepare 5 mL of XF assay medium for each 8-well XF HS miniplate and warm it up in a 37°C water bath.
d. Aspirate the fibroblast media from the XF HS miniplate containing fibroblasts without disturbing the cells.
e. Gently wash out each well of the XF HS miniplate twice with 180 µL of XF assay medium.
f. Add 180 µL of XF assay medium into each well of the XF HS miniplate and put the plate into the non-CO2 37°C incubator for 1 h.

4. Preparing compounds for loading sensor cartridge.
   a. After 20 min, reconstitute compounds with pre-warmed XF assay medium.
   b. Dilute the reconstituted compounds with pre-warmed XF assay medium.

   Note: We suggest using 2 µM FCCP as a final concentration. However, the optimal dose of FCCP may vary depending on cell context. Determine and identify the optimal FCCP concentration to provoke maximal respiration of your cells.

c. Move a hydrated sensor cartridge assembly from the non-CO2 37°C incubator to the laminar flow hood and load the diluted compounds into the indicated ports of the hydrated sensor cartridge.
   i. Add 20 µL of diluted oligomycin solution into Port A of the hydrated sensor cartridge.
   ii. Add 22 µL of diluted FCCP solution into Port B of the hydrated sensor cartridge.
   iii. Add 25 µL of diluted rotenone/antimycin A solution into Port C of the hydrated sensor cartridge.

   Note: Port D of the hydrated sensor cartridge should be empty unless users want to test their own chemicals.

d. Hit ‘START’ and run the ‘Mito Stress Test’ program of the XF HS Mini Analyzer.
e. Place the compounds-loaded, hydrated sensor cartridge assembly onto the load position of XF HS Mini Analyzer.

   Note: See the user manual for operation details of the XF HS Mini Analyzer (https://www.agilent.com/cs/library/usermanuals/public/user-manual-flux-analyzer-xf-hs-mini-extracellular-5994-1961en-agilent.pdf).

5. Running metabolic analysis.
   a. Once the calibration step of the hydrated sensor cartridge is completed, switch the Utility plate with the XF HS miniplate containing cells and click ‘CONTINUE’.

6. Determining protein concentration.
   a. Once the assay is completed, take out the assayed XF HS miniplate from the cartridge-miniplate assembly and carefully aspirate the medium from each well of the miniplate without disturbing the attached cells.
   b. Wash out the cells with 200 µL of DPBS once.
   c. After aspirating DPBS, add 200 µL of Bradford dye reagent into each well of the miniplate and mix well to prepare a crude cell lysate.
   d. Measure each sample optical density with a spectrometer to assess the protein concentration.

   Alternatives: In this protocol, we used a Synergy HTX multi-mode reader. However, spectrometers from different providers also work with this protocol.

7. Analyzing data.
   a. Analyze raw data using the Seahorse WAVE Desktop software.
b. The following factors can be presented as a result of real-time metabolic analysis.
   i. Oxygen consumption rate (OCR; pmol/min/µg protein).
   ii. Extracellular acidification rate (ECAR; mpH/min/µg protein).
   iii. OCR/ECAR ratio.
   iv. Basal respiration = baseline OCR – rotenone/antimycin A OCR.
   v. Maximal respiration = FCCP OCR – rotenone/antimycin A OCR.
   vi. ATP turnover = baseline OCR – oligomycin OCR.
   vii. Oxidative reserve = maximal respiration – basal respiration.
   viii. OCR changes after FCCP injection.

Quantification of mitochondrial network

© Timing: 3 days

Below we provide a detailed step-by-step protocol for the quantification of mitochondrial network by immunostaining analysis.

8. Preparing samples.
   a. Plate fibroblasts onto the 4-chamber cell culture slide at 2.5 x 10^5 cells/well (~80% confluency) and incubate in a 5% CO₂ 37°C incubator for 18–24 h.
   b. Next day, wash out the chamber slide containing fibroblasts with 1 mL of DPBS twice.
   c. Aspirate the DPBS and fix the cells for 10 min with 0.5 mL of 10% formaldehyde solution.
   d. Aspirate the formaldehyde solution and wash out the cells three times for 10 min with 1 mL of DPBS.

Pause point: If you do not have enough time to stain the cells, fill the chamber slide with 1 mL of DPBS, seal with parafilm, and store it at 4°C for up to 1 week.

   e. Aspirate the DPBS and incubate the cells for 30 min with 1 mL of blocking solution.
   f. Aspirate the blocking solution and incubate the cells with anti-TOM20 antibody in blocking solution at 4°C for 18–24 h on a shaker.
   g. Next day, aspirate the diluted antibody solution and wash the cells three times for 10 min with 1 mL of washing solution.
   h. Aspirate the washing solution and incubate the cells for 1 h on a shaker at room temperature (15°C–30°C) with anti-mouse secondary antibody in blocking solution.
   i. Aspirate the diluted antibody solution and wash the cells three times for 10 min with 1 mL of washing solution.
   j. Aspirate the washing solution and incubate the cells for 5 min with 500 µL of Hoechst 33342 in DPBS at room temperature (15°C–30°C).
   k. Aspirate the diluted Hoechst 33342 solution, wash the cells three times for 10 min with 1 mL of DPBS.
   l. Aspirate the DPBS, remove the chamber from the slide.
   m. Apply one drop of Fluoroshield solution onto the slide and cover it with a microscope cover glass.
   n. Once the Fluoroshield is dry, image the slide under a fluorescence microscope.

9. Quantifying mitochondrial network.
   a. Download and install software including the Fiji distribution of ImageJ2 software (https://imagej.net/software/fiji/) and the Mitochondrial Network Analysis (MiNA; https://github.com/StuartLab/MiNA) toolset.
b. Perform the ‘MiNA Analyze Morphology’ script by navigating to the ‘StuartLab’ under the ‘Plugins’ menu of the Fiji software.

c. Process the acquired image with the ‘MiNA Analyze Morphology’ script as described below (Figure 1):
   i. Set up ‘Unsharp Mask’ as 1st place.
   ii. Set up ‘Enhance Local Contrast CLAHE’ as 2nd place.
   iii. Set up ‘Median Filter’ as 3rd place.
   iv. Select ‘Preview Preprocessing’ function.
   v. Perform the script by pressing the ‘OK’ button.

d. The following factors can be quantified from the parameters released from processing with MiNA.
   i. Mitochondrial footprint (µm²).
   ii. Summed branch length (µm).

Assessment of mitochondrial mass

© Timing: 2 h

Below we provide a detailed step-by-step protocol on how to perform the measurement of mitochondrial mass by flow cytometry analysis. We recommend adding CCCP as a positive control for assessing mitochondrial mass.

10. Preparing samples.
   a. Collect the cells grown on 6 cm tissue culture dish at ~80% confluency (up to 1 × 10⁶ cells) by using TrypLE™ express enzyme solution.
b. Spin down the cells at 200 × g for 3 min.
c. Aspirate the medium and wash the cells with 1 mL of DPBS twice.
d. Aspirate the DPBS and incubate the cells with MitoTracker™ Green FM (150 nM in DPBS) for 25 min at 37°C.
e. Spin down the cells at 200 × g for 3 min.
f. Aspirate the MitoTracker™ Green FM solution and wash the cells with 1 mL of DPBS twice.
g. Reconstitute the cells with 1 mL of DPBS.

11. Measuring mitochondrial mass.
   a. Measure the mitochondrial mass using a MACSQuant® Analyzer 16.

   **Note:** See the user manual for details to operate the MACSQuant® Analyzer 16 (https://www.miltenyibiotec.com/_Resources/Persistent/931639a5b475f080764f13e26d034cbb857a1c9c/MACSQuant%20Instrument%20User%20Manual.pdf).

b. Analyze the raw data using FlowJo.

   **Note:** See the user manual for details to analyze flow cytometry data using FlowJo (https://f.hubspotusercontent20.net/hubfs/2566672/Marketing/Flowjo_Introduction.pdf).

c. The following factors can be presented based on assessment of mitochondrial mass.
   i. Mean fluorescence intensity (MFI).

**Assessment of mitochondrial membrane potential**

© Timing: 2 h

Below we provide a detailed step-by-step protocol on how to measure mitochondrial membrane potential by flow cytometry analysis.

12. Preparing samples.
   a. Collect the cells grown on 6 cm tissue culture dish at ~80% confluency (up to 1 × 10⁶ cells) by using TryPLE™ express enzyme solution.
   b. Spin down the cells at 200 × g for 3 min.
   c. Aspirate the medium and wash the cells with 1 mL of DPBS twice.
   d. Aspirate the DPBS and incubate the cells with JC-1 cationic dye (10 µg/mL in DPBS) for 10 min at 37°C.
   e. Spin down the cells at 200 × g for 3 min.
   f. Aspirate the JC-1 solution and wash the cells with 1 mL of DPBS twice.
   g. Reconstitute the cells with 1 mL of DPBS.

13. Measuring mitochondrial membrane potential.
   a. Measure the mitochondrial membrane potential using a MACSQuant® Analyzer 16.

   **Note:** See the user manual for details to operate the MACSQuant® Analyzer 16 (see above).

b. Analyze the raw data using FlowJo.

   **Note:** See the user manual for details to analyze flow cytometry data using FlowJo (see above).

c. The following factors can be presented as a result of assessment of mitochondrial membrane potential.
i. Ratio of green fluorescence (emitted at 525 nm by JC-1 monomers) vs. red fluorescence (emitted at 590 nm by JC-1 aggregates).

**Measurement of mitochondrial metabolites**

© Timing: 2–3 h

Below we provide a detailed step-by-step protocol on measuring mitochondrial metabolites by using metabolite-specific colorimetric assay kit (all from BioVision).

**Note:** See the user manual for details on how to perform the mitochondrial metabolites assay.

14. Preparing samples.
   a. Collect the cells grown on 6 cm tissue culture dish at ~80% confluency (up to $1 \times 10^6$ cells) by using a cell lifter.
   b. Transfer the cell suspension into a 15 mL conical tube and spin down the cells at 200 \( \times \) g for 3 min.
   c. Aspirate the medium and resuspend the cells with 1 mL of DPBS.
   d. Transfer the cell suspension into a 1.5 mL microcentrifuge tube and spin down the cells at 6,000 \( \times \) g for 1 min.
   e. Aspirate the DPBS and lyse the cells with 100 
   mL \( \times \) Cell Lysis buffer on ice for 30 min.

**Alternatives:** For the lysis step, 1 \( \times \) RIPA buffer (Cat# 9806; Cell Signaling Technologies) can be used instead of 1 \( \times \) Cell Lysis buffer.

f. Centrifuge the lysed cells at 15,000 \( \times \) g, 4°C for 10 min.

**Pause point:** Cell lysates can be stored at \(-20^\circ C\) until use.

g. Transfer the lysate carefully into new 1.5 mL microcentrifuge tube without disturbing cell pellet and store the samples on ice.

h. Measure the protein concentration of samples with a spectrometer using a Bradford assay.

15. Measuring mitochondrial metabolites.
   a. Add 0, 2, 4, 6, 10 \( \mu \)L of diluted standard solution into a series of standards wells on a 96-well microplate.
   b. Add 50, 48, 46, 44, 42, 40 \( \mu \)L of Assay buffer into the wells of the 96-well microplate containing the standards to adjust the volume to 50 \( \mu \)L/well.
   c. Add 1–50 \( \mu \)L of samples into duplicate wells of the 96-well microplate.
   d. Add Assay buffer into the sample wells of the 96-well microplate to adjust the volume to 50 \( \mu \)L/well.
   e. Add 50 \( \mu \)L of the Reaction mix solution to each well of the 96-well microplate containing the standards and samples.
   f. Incubate the microplate in a non-CO\(_2\) 37°C incubator for 30 min.
   g. Measure the OD values of samples on a microplate reader at appropriate wavelengths as described below:
      i. 450 nm for malate.
      ii. 570 nm for citrate, \( \alpha \)-ketoglutarate, and oxaloacetate.
   h. For kinetic readings, repeat the microplate incubation at 37°C and measure the OD values at 20 min intervals up to 1 h.

16. Analyzing data.
   a. Calculate metabolite concentrations of each sample from the recorded OD values for the standard solution and samples as below:
      i. \( C = \frac{A_y}{S_y} \) (nmol/\( \mu \)g protein).
**EXPECTED OUTCOMES**

You can determine the effect of interested genes on mitochondrial oxidative metabolism in human fibroblasts via either pharmacological inhibition or genetic manipulation. In our recent study, we
adopted this integrated analysis method to determine the functional roles of SIRT2, one of the NAD+-dependent class III histone deacetylases, on mitochondrial oxidative metabolism in human fibroblasts (Cha et al., 2021). SIRT2 is highly enriched in somatic cells including fibroblasts that mainly depend on mitochondrial oxidative metabolism and is consistently downregulated during the somatic cell reprogramming process (Cha et al., 2017). SIRT2 knockdown (KD) in fibroblasts significantly reduces mitochondrial OXPHOS capacity compared to control cells, as evidenced by decreases in basal respiration, maximal respiration, ATP turnover, and oxidative reserve, as well as OCR changes after FCCP injection (Figure 2). In addition, SIRT2KD in fibroblasts results in shortened mitochondrial length, compared to control cells (Figure 3). Furthermore, reduced mitochondrial membrane potential and mitochondrial mass can be detected in SIRT2KD fibroblasts compared with control fibroblasts (Figures 4 and 5). Moreover, the levels of mitochondrial TCA cycle metabolites, including citrate, α-KG, malate, and OAA are significantly reduced by SIRT2KD (Figure 6), demonstrating that SIRT2 controls mitochondrial oxidative metabolism in human fibroblasts via regulating mitochondrial dynamics.

This protocol is also applicable to assess the mitochondrial metabolic dynamics in iPSCs that go through differentiation or altered pluripotency (e.g., naïve and primed pluripotency).

LIMITATIONS

In this experiment, we have used two human fibroblast lines, including BJ newborn dermal fibroblasts and GM03529 adult dermal fibroblasts. Therefore, to perform these experiments with another fibroblast lines, users need to test and optimize the experimental conditions for their own cells.

TROUBLESHOOTING

Problem 1

OCR and ECAR signals from Seahorse XF assay are too low to analyze (step 7 in step-by-step method details).

Potential solution

This is mainly due to low plated cells’ density. Plate the cells onto XF HS miniplate at over 90% confluence. The other possibility is the existence of bubbles on the surface of the XF HS miniplate during the cell plating step (step 1 in step-by-step method details). To avoid this, check the bottom of the miniplate after plating the cells.
Problem 2

Minor change in graph from Seahorse XF assay after FCCP injection (step 7 in step-by-step method details).

Potential solution

This is mainly due to low FCCP concentration. Adjust the FCCP concentration (up to 2 μM) to identify an optimal concentration for your cells.

Figure 4. Representative results obtained from mitochondrial membrane potential analysis

Sample results obtained using mitochondrial membrane potential analysis shown in (Cha et al., 2021).

(A) Representative flow cytometry images from mock and SIRT2KD BJ hDFs stained with JC-1 fluorescent dye. CCCP as positive control.

(B) Quantification of the fluorescence ratio of JC-1 (green/red) from wild-type (hDF), mock, and SIRT2KD BJ hDFs (n = 6). Data are represented as mean ± SD, **p<0.01, One-way ANOVA with Tukey's post-test calculated using Prism 8 software. Published with permission from Elsevier.

Figure 5. Representative results obtained from mitochondrial mass analysis

Sample results obtained using mitochondrial mass analysis.

(A) Representative flow cytometry images from mock and SIRT2KD BJ hDFs stained with MitoTracker™ Green FM fluorescent dye. CCCP as positive control.

(B) Quantification of the Mean Fluorescence Intensity of MitoTracker™ Green FM from wild-type (hDF), mock, and SIRT2KD hDFs (n = 6). Data are represented as mean ± SD, ***p<0.005, ****p<0.001, One-way ANOVA with Tukey’s post-test calculated using Prism 8 software.
Problem 3
Poor attachment of fibroblasts on chamber slide stained with anti-TOM20 antibody (step 8 in step-by-step method details).

Potential solution
Pre-coat the chamber slide with Matrigel solution before plating the cells. This will increase the strength of cell attachment.

Problem 4
Poor mitochondrial metabolite assay signals (step 15 in step-by-step method details).

Potential solution
Increase the amount of sample lysate added to the wells of the 96-well microplate.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kwang-Soo Kim (kskim@mclean.harvard.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Original/source data for the figures presented in this paper are available at [https://doi.org/10.1016/j.celrep.2021.110155].

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AUTHOR CONTRIBUTIONS
Conceptualization and writing – original draft, Y.C. and K.-S.K.; methodology and investigation, Y.C.; writing – review & editing, Y.C., P.L., Y.J.H., and K.-S.K.; funding acquisition and supervision, K.-S.K.
DECLARATION OF INTERESTS
All authors declare no competing interests.

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