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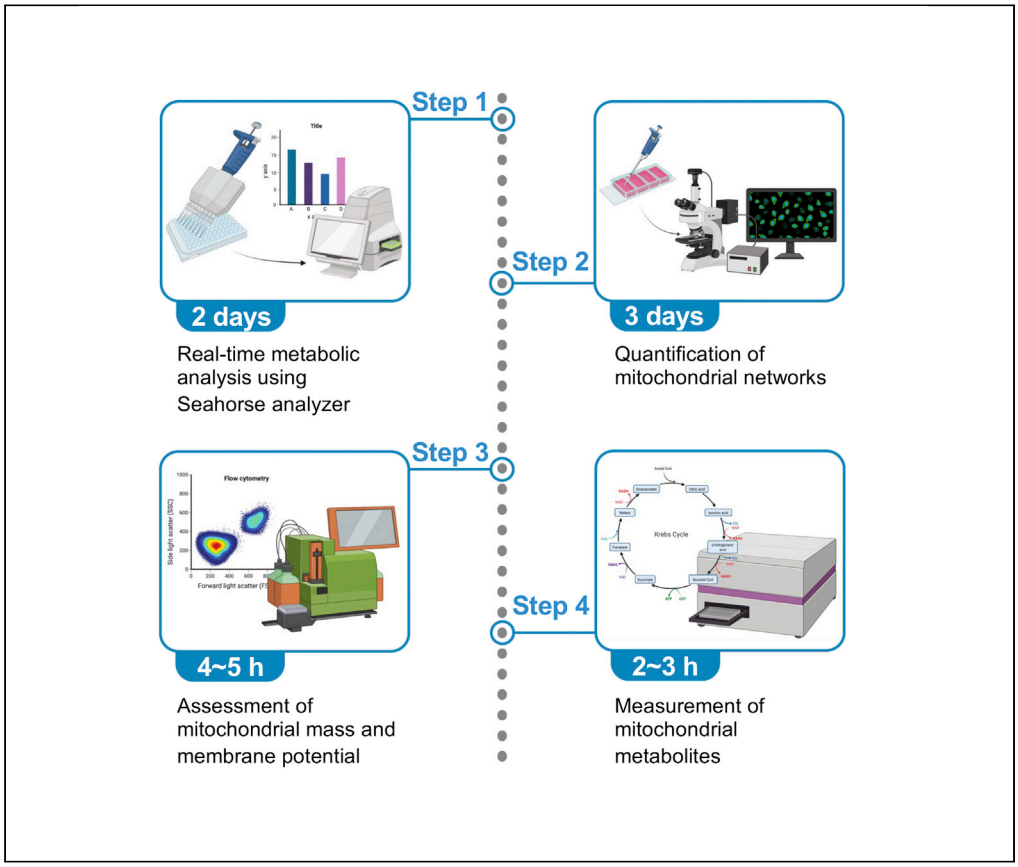
Protocol

Integrative analysis of mitochondrial metabolic dynamics in reprogramming human fibroblast cells



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.

Young Cha, Pierre Leblanc, Yean Ju Hong, Kwang-Soo Kim

ycha@mclean.harvard.edu (Y.C.)
kskim@mclean.harvard.edu (K.-S.K.)

Highlights

Protocol for integrative analysis of fibroblast mitochondrial metabolic dynamics

Step-by-step guide for assessing different parameters of mitochondrial function

Flow cytometry-based analysis of mitochondrial mass and membrane potential

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Integrative analysis of mitochondrial metabolic dynamics in reprogramming human fibroblast cells

Young Cha,[1,2,*] Pierre Leblanc,[1] Yean Ju Hong,[1] and Kwang-Soo Kim[1,3,*]

[1]Department of Psychiatry and Molecular Neurobiology Laboratory, McLean Hospital and Program in Neuroscience, Harvard Medical School, Belmont, MA 02478, USA

[2]Technical contact

[3]Lead contact

*Correspondence: ycha@mclean.harvard.edu (Y.C.), kskim@mclean.harvard.edu (K.-S.K.)
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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% CO_2 and non-CO_2 37°C incubators are required for performing the protocol operations. Furthermore, users need to have ready access to key equipment, including a Seahorse XF HS 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% CO_2 37°C incubator.
j. Cells should be fed with 8 mL of pre-warmed fresh fibroblast media every other day until confluency 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% CO_2 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% CO_2 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% CO_2 37°C incubator.
y. From next day on, feed cells with 3 mL of NutriStem® hPSC 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		
Seahorse XF calibrant solution	Agilent Technologies	Cat# 103059-100
Seahorse XF base medium, without phenol red	Agilent Technologies	Cat# 103335-100

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Quick Start™ Bradford 1× dye reagent	Bio-Rad Laboratories	Cat# 5000205
Cell Lysis Buffer (10×)	Cell Signaling Technology	Cat# 9803
Matrigel® hESC-Qualified Matrix, LDEV-free	Corning	Cat# 354277
NutriStem® hPSC XF medium	Sartorius	Cat# 05-100-1A
Y-27632	Selleckchem	Cat# S1049
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Selleckchem	Cat# S6494
Formaldehyde solution	Sigma-Aldrich	Cat# 252549
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# D2650
Fluoroshield™ histology mounting medium	Sigma-Aldrich	Cat# F6182
D-glucose solution	Sigma-Aldrich	Cat# G8769
Triton X-100	Sigma-Aldrich	Cat# T9284
DMEM	Thermo Fisher Scientific	Cat# 11965092
DMEM/F12	Thermo Fisher Scientific	Cat# 11320082
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 26140079
GlutaMAX™ supplement	Thermo Fisher Scientific	Cat# 35050061
MEM non-essential amino acids (NEAA) solution	Thermo Fisher Scientific	Cat# 11140050
Sodium pyruvate solution (100 mM)	Thermo Fisher Scientific	Cat# 11360070
β-mercaptoethanol (β-ME)	Thermo Fisher Scientific	Cat# 21985023
DPBS	Thermo Fisher Scientific	Cat# 14190144
TrypLE™ express enzyme	Thermo Fisher Scientific	Cat# 12605028
Trypan blue solution (0.4%)	Thermo Fisher Scientific	Cat# 15250061
Hoechst 33342 (used at 1:5,000 dilution)	Thermo Fisher Scientific	Cat# H3570
Normal horse serum blocking solution	Vector Laboratories	Cat# S-2000-20
Critical commercial assays		
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
Oxaloacetate 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# T3168
MitoTracker™ Green FM	Thermo Fisher Scientific	Cat# M7514
Experimental models: Cell lines		
Human BJ newborn dermal fibroblasts	ATCC	Cat# CRL-2522; RRID: CVCL_3653
Human adult dermal fibroblasts	Coriell Institute	Cat# GM03529; RRID: CVCL_7394
Recombinant DNA		
pCXLE-OKSlM	(Song et al., 2020)	N/A
pCXLE-miR-302s/200c	(Song et al., 2020)	N/A
pCXWB-EBNA1	Addgene	Cat# 37624
Software and algorithms		
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)	https://github.com/StuartLab/MiNA
Other		
Seahorse XF HS Mini Analyzer	Agilent Technologies	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers/seahorse-xf-hs-mini-analyzer-770502
Seahorse XFp FluxPak	Agilent Technologies	Cat# 103022-100
BD Luer-Lok™ 50 mL syringe	Becton Dickinson & Company	Cat# 309653
Synergy HTX multi-mode reader	BioTek Instruments	https://www.biotek.com/products/detection-multi-mode-microplate-readers/synergy-htx-multi-mode-reader/overview/

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sterile micropipette tips (10, 20, 200, 1,000 µL)	CELLTREAT Scientific Products	Cat# 229015; 229017; 229019; 229021
Serological pipettes (5, 10, 25 mL)	CELLTREAT Scientific Products	Cat# 229005B; 229010B; 229025B
6-well tissue culture plate	CELLTREAT Scientific Products	Cat# 229106
4-well chamber cell culture slide	CELLTREAT Scientific Products	Cat# 229164
Cell lifter	CELLTREAT Scientific Products	Cat# 229305
500 mL filter system	CELLTREAT Scientific Products	Cat# 229707
Tissue culture dishes (6 cm)	Corning Incorporated	Cat# 353004
Sterile conical tubes (15, 50 mL)	Corning Incorporated	Cat# 352096; 352098
96-well microplate	Corning Incorporated	Cat# 3370
Pipette aid	Drummond Scientific	Cat# 4-000-101
Millex-GV syringe filter unit (0.22 µm)	EMD Millipore	Cat# SLGVM33RS
8-channel micropipette (30–300 µL)	Fisher Scientific	Cat# FBE800300
Micropipettes (10, 20, 200, 1,000 µL)	Gilson Incorporated	Cat# FA10002M; FA10003M; FA10005M; FA10006M
Bright-Line Hemacytometer	Hausser Scientific	Cat# 1492
Microscope cover glass	Kemtech America	Cat# 0341-3650
All-in-One fluorescence microscope	Keyence Corporation	BZ-X700 or equivalent
MACSQuant® Analyzer 16 flow cytometer	Miltenyi Biotec	https://www.miltenyibiotec.com/US-en/products/macsquant-analyzer-16.html#gref
CO_2 incubator	Sanyo Scientific	Cat# MCO-19AIC
Orbital shaker	Scilogex	Cat# SCI-O180-S
NUNC™ T-25 cell culture flask	Thermo Fisher Scientific	Cat# 156367
Neon™ Transfection System	Thermo Fisher Scientific	Cat# MPK5000
1.5 mL microcentrifuge tube	USA Scientific	Cat# 1615-5510

MATERIALS AND EQUIPMENT

Fibroblast medium		
Reagents	Final concentration	Amount
DMEM	1×	419.5 mL
FBS	15%	75 mL
NEAA (100× solution)	1×	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
pCXWB-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.

Plating medium		
Reagents	Final concentration	Amount
DMEM	1×	41.95 mL
FBS	15%	7.5 mL
NEAA (100× solution)	1×	500 μL
Y-27632	10 μM	50 μL
Total	n/a	50 mL

- Filter the medium with a 0.22 μm filter unit.
- Plating medium should be freshly prepared for every new experiment.

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

- XF assay medium should be freshly prepared in the laminar flow hood for every new experiment.
- Warm up the XF assay medium in 37°C water bath at atmospheric CO_2 conditions before use.

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.

- Reconstituted compounds should be used on the same day.

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 (25 μM solution)	0.5 μM	Mix 60 μL of stock solution with 240 μL of XF assay medium and vortex thoroughly.

- 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™ 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 solution	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) converting enzyme	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.

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Reagents	Stock solution 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	1×	Add 220 μL of malate buffer to stock vial and vortex thoroughly.
WST substrate	1×	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	1×	Add 220 μL of OAA buffer to stock vial and vortex thoroughly.
OAA developer	1×	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.

- Store stock solutions for mitochondrial metabolite assay at −20°C for up to 2 months.

1× Reaction Mix solution for citrate assay

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

- 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.

1× Reaction Mix solution for α-KG assay

Reagents	Final concentration	Amount per sample well	Amount per standard well
α-KG assay buffer	1×	44 μL	46 μL
α-KG converting enzyme mix	1×	2 μL	–
α-KG development enzyme mix	1×	2 μL	2 μL
α-KG probe	1×	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.

1× Reaction Mix solution for malate assay

Reagents	Final concentration	Amount per well
Malate assay buffer	1×	38 μL
Malate enzyme mix	1×	2 μL
WST substrate	1×	10 μL
Total	**n/a**	**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.

1× Reaction Mix solution for OAA assay			
Reagents	Final concentration	Amount per sample well	Amount per standard well
OAA assay buffer	1×	44 μL	46 μL
OAA enzyme mix	1×	2 μL	–
OAA developer	1×	2 μL	2 μL
OAA probe	1×	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 × 10^3 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% CO_2 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-CO_2 37°C incubator for 18–24 h.

⚠ CRITICAL: The non-CO_2 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-CO_2 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-CO_2 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.

Note: See the user manual for operation details of the WAVE software (https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_C_Wave_2_6_User_Guide.pdf).

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 2.5~3 × 10^5 cells/well (~80% confluency) and incubate in a 5% CO_2 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.

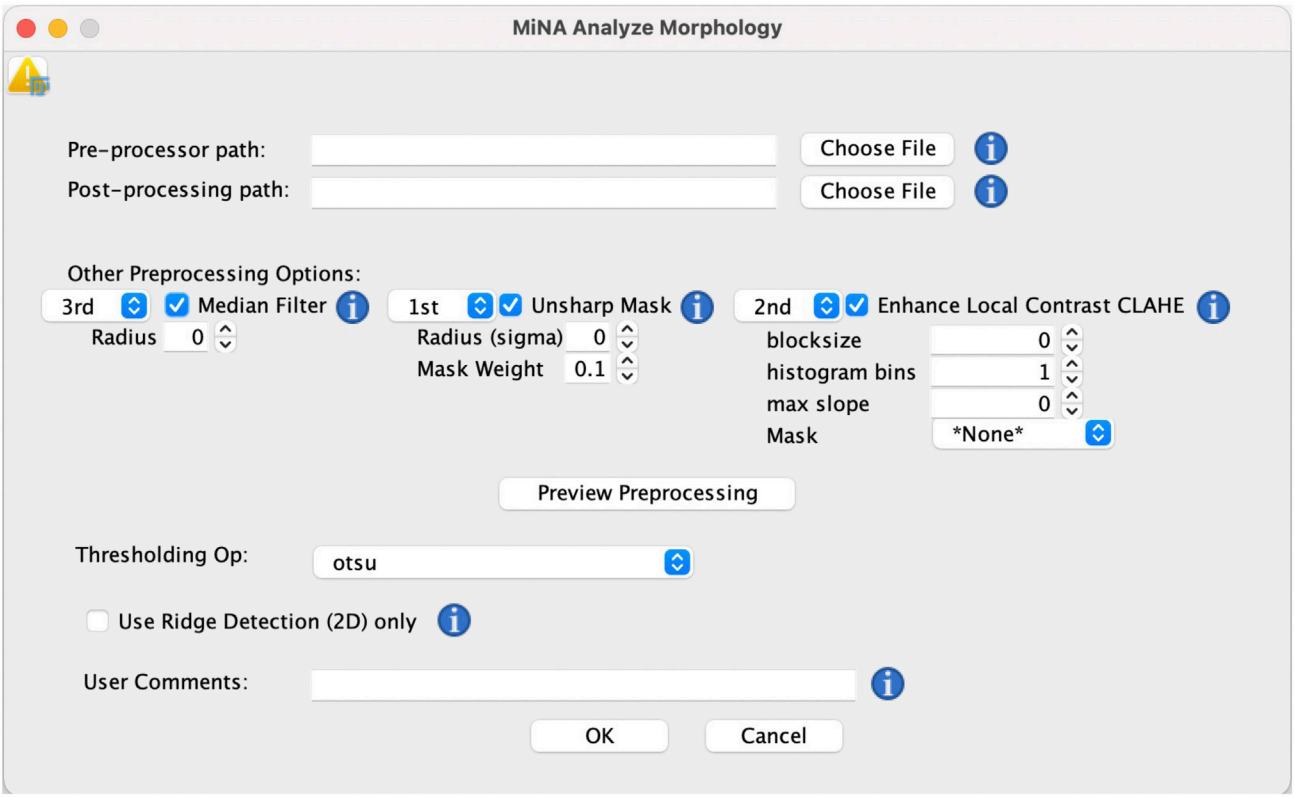

Figure 1. Setting up the 'MiNA Analyze Morphology' plugin for quantifying mitochondrial network

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 (μm^2).
   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 1×10^6 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 × 10^6 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 × 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 × *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 × *g* for 1 min.
    e. Aspirate the DPBS and lyse the cells with 100 μL of 1× Cell Lysis buffer on ice for 30 min.

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

    f. Centrifuge the lysed cells at 15,000 × *g*, 4°C for 10 min.
    g. Transfer the lysate carefully into new 1.5 mL microcentrifuge tube without disturbing cell pellet and store the samples on ice.

Pause point: Cell lysates can be stored at −20°C until use.

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

15. Measuring mitochondrial metabolites.
    a. Add 0, 2, 4, 6, 8, 10 μL of diluted standard solution into a series of standards wells on a 96-well microplate.
    b. Add 50, 48, 46, 44, 42, 40 μL of Assay buffer into the wells of the 96-well microplate containing the standards to adjust the volume to 50 μL/well.
    c. Add 1–50 μL 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 μL/well.
    e. Add 50 μL 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, α-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 = Ay / Sy (nmol/μg protein).

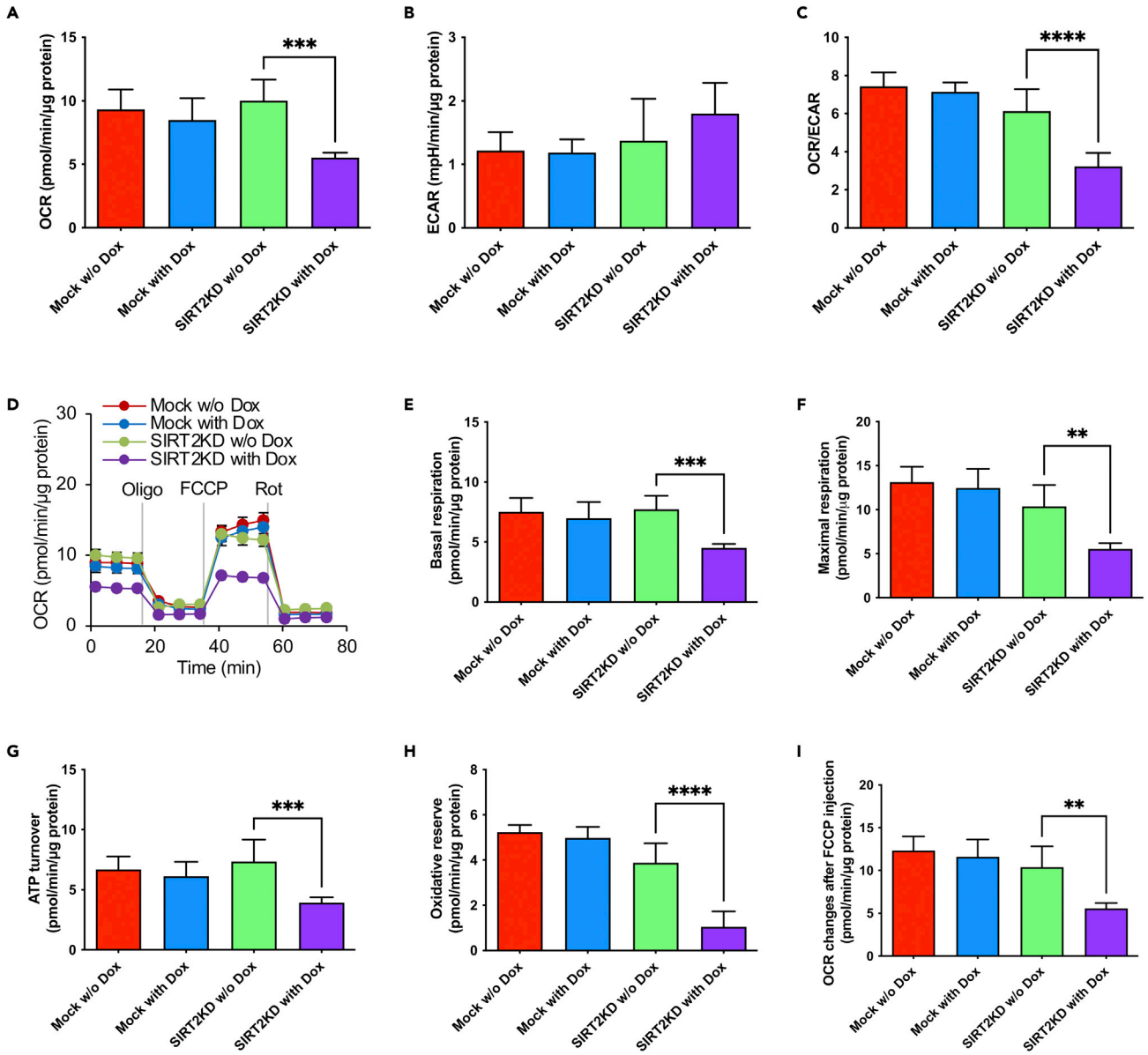

Figure 2. Representative results obtained from real-time metabolic analysis

Sample results obtained using real-time metabolic analysis shown in (Cha et al., 2021).

(A–C) Oxygen consumption rate (OCR) (A), extracellular acidification rate (ECAR) (B) and OCR/ECAR ratio (C) of human fibroblasts (hDFs) treated with control (mock) or specific shRNA (KD) against SIRT2 in a doxycycline (Dox)-inducible manner (n = 5).

(D) Comparison of OXPHOS capacity of mock and SIRT2KD at 2 days after Dox treatment (n = 5).

(E–I) Comparison of basal respiration (E), maximal respiration (F), ATP turnover (G), oxidative reserve (H), and OCR changes after FCCP injection (I) from mock and SIRT2KD, as shown in (D) (n = 5). Data are represented as mean ± SD, **p<0.01; ***p<0.005; ****p<0.001, One-way ANOVA with Tukey's post-test calculated using Prism 8 software. Published with permission from Elsevier.

ii. Ay: amount of metabolite (nmol) in samples from the standard curve.

iii. Sy: amount of samples (μg) added to the sample well.

b. The data can be presented as heatmaps, scatter plot or bar charts.

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

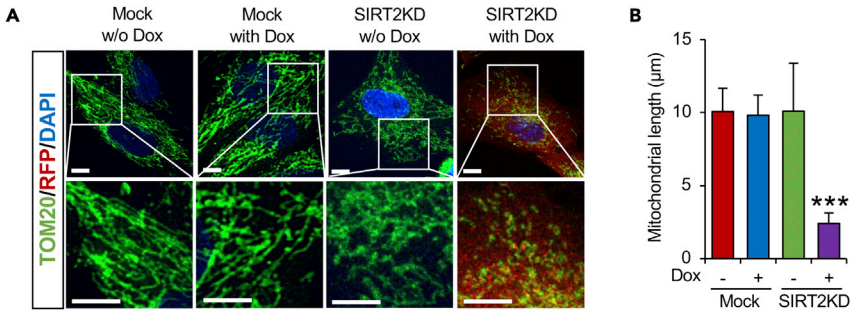

Figure 3. Representative results obtained from mitochondrial network analysis

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

(A) Representative immunofluorescent images of TOM20 from inducible SIRT2KD hDFs with or without Dox. Scale bar, 10 μm.

(B) Quantification of mitochondrial length between mock and SIRT2KD (n = 15). Data are represented as mean ± SD, ***p<0.005, One-way ANOVA with Tukey's post-test calculated using Prism 8 software. Published with permission from Elsevier.

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% confluency. 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.

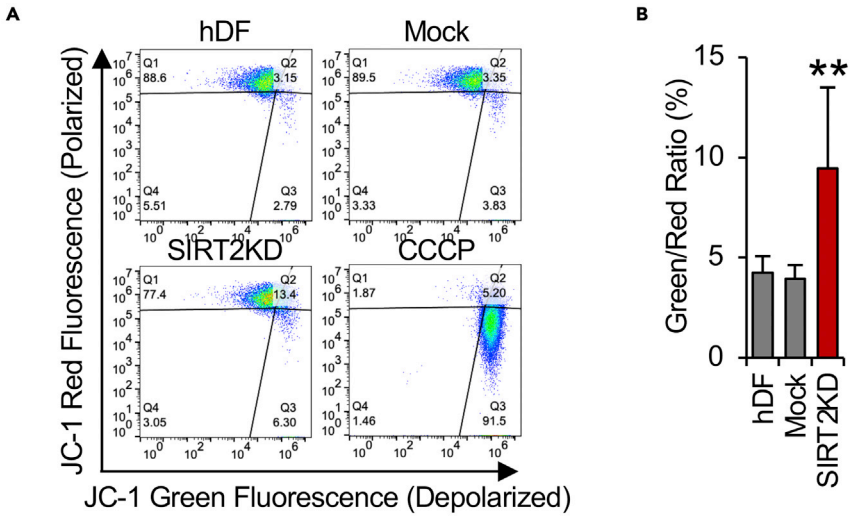

Figure 4. Representative results obtained from mitochondrial membrane potential analysis

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.

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.

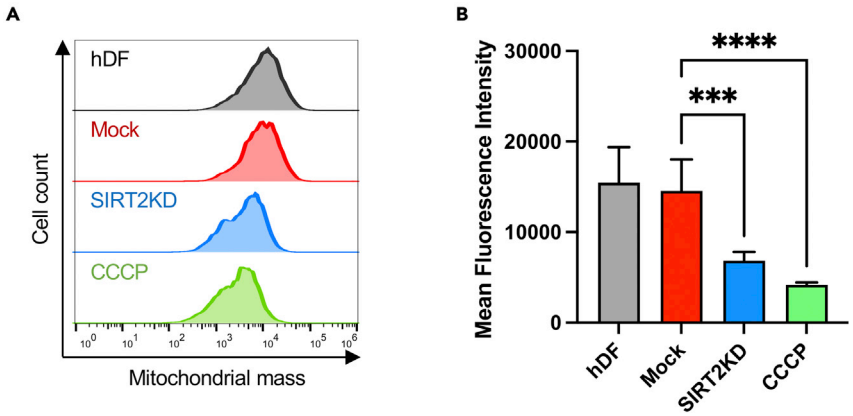

Figure 5. Representative results obtained from mitochondrial mass analysis

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.

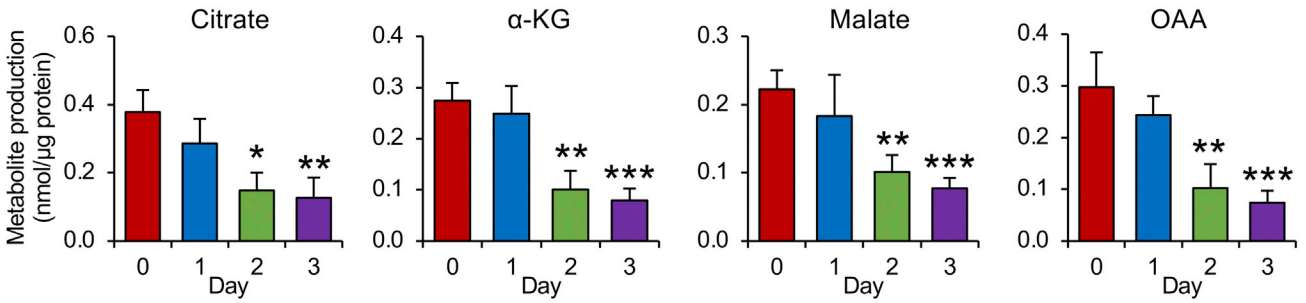

Figure 6. Representative results obtained from mitochondrial metabolites assay

Sample results obtained using mitochondrial metabolites assay shown in (Cha et al., 2021). Production of key metabolites, including citrate, α-KG, malate and OAA were assessed from SIRT2KD hDFs (n = 4). Data are represented as mean ± SD, *p<0.05; **p<0.01; ***p<0.005, One-way ANOVA with Tukey's post-test calculated using Prism 8 software. Published with permission from Elsevier.

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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