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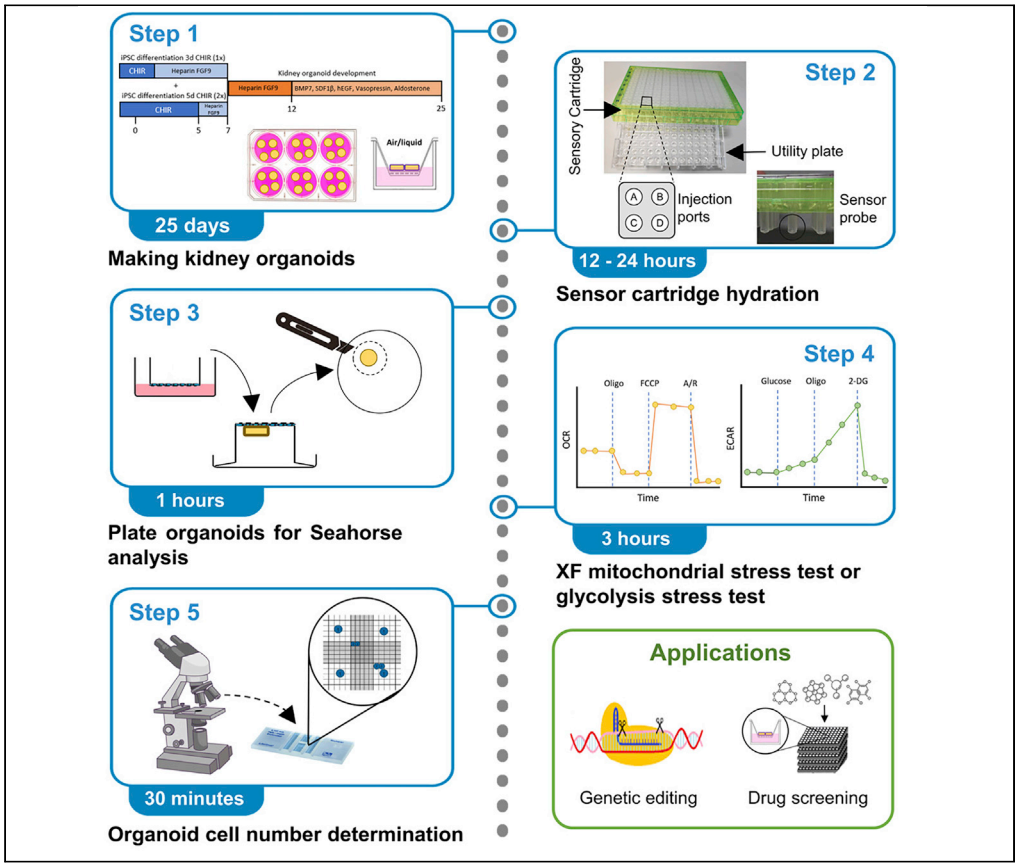
Protocol to analyze bioenergetics in single human induced-pluripotent-stem-cell-derived kidney organoids using Seahorse XF96



Metabolic derangement is a key culprit in kidney pathophysiology. Organoids have emerged as a promising *in vitro* tool for kidney research. Here, we present a fine-tuned protocol to analyze bioenergetics in single human induced-pluripotent-stem-cell (iPSC)-derived kidney organoids using Seahorse XF96. We describe the generation of self-organized three-dimensional kidney organoids, followed by preparation of organoids for Seahorse XF96 analysis. We then detail how to carry out stress tests to determine mitochondrial and glycolytic rates in single kidney organoids.

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

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Highlights

Generation of self-organized three-dimensional kidney organoids

Coupling organoid generation with Seahorse technology

Determination of mitochondrial and glycolytic rates in single kidney organoids

Bioenergetic normalization by organoid cell number to overcome individual variabilities

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SUMMARY

Metabolic derangement is a key culprit in kidney pathophysiology. Organoids have emerged as a promising *in vitro* tool for kidney research. Here, we present a fine-tuned protocol to analyze bioenergetics in single human induced-pluripotent-stem-cell (iPSC)-derived kidney organoids using Seahorse XF96. We describe the generation of self-organized three-dimensional kidney organoids, followed by preparation of organoids for Seahorse XF96 analysis. We then detail how to carry out stress tests to determine mitochondrial and glycolytic rates in single kidney organoids.

BEFORE YOU BEGIN

The kidney has a complex architectural pattern whose highly differentiated cells exhibit diverse metabolic specialization to fulfill energy requirements for blood filtration, salt-water balance and electrolyte homeostasis. Thus, metabolic heterogeneity is an important aspect of kidney development and physiology.[1] Metabolic reprogramming has recently emerged as an integral indicator of developmental abnormalities and disease susceptibility.[2,3] Human induced pluripotent stem cell (iPSC)-derived organoids represent a three-dimensional kidney system that contains various renal compartments and cell types.[4] They constitute a useful tool to model kidney diseases and to validate therapeutic targets.[5,6] The analysis of the kidney's bioenergetics is essential to figure out, and potentially target, the metabolic changes underlying such processes. Adenosine triphosphate (ATP) is the key energy-carrying molecule at cellular level. Oxidative phosphorylation in the mitochondria and glycolysis in the cytosol generate ATP at different rates and with distinct requirements and side-products.[7] Oxygen consumption rate (OCR) (ligated to oxidative phosphorylation-linked mitochondrial respiration) and extracellular acidification rate (ECAR) (associated with lactate production-linked glycolysis) are the read-outs reported by the Seahorse analyzer.[8] Changes in OCR and ECAR measured in the presence of different metabolic substrates and inhibitors serve to calculate several bioenergetic parameters. Here, we describe adapted protocols for mitochondrial and glycolysis stress tests that allow metabolic profiling of single differentiated kidney organoids under disease conditions or following genetic or pharmacologic perturbation.

Institutional permissions

Consider potential institutional permissions for the generation and handling of reprogrammed iPSCs. The iPSCs line used in the current study (iPS134) were generated from human erythroblasts from a pediatric patient using the Yamanaka factors[9] by the Stem Cells Technology Center at Radboud University Medical Center (SCTC, Radboud UMC, Nijmegen, The Netherlands). The patient is a carrier of a heterozygous mutation in the NPHS2 (podocin) gene. This study was conducted in accordance with the Helsinki Declaration as revised in 2013. Permission for the creation and use of iPSCs in this study was obtained from the local ethical commission for human-related research of the Radboud UMC, Nijmegen (approval numbers: 2015-1543 and 2006-048). We are working under a Material Transference Agreement (MTA) between Rafael Kramann's laboratory at RWTH Aachen University and Radboud UMC for using iPSC.

General laboratory preparation

1. Perform iPSC and kidney organoid culture experiments in a biosafety level 1 flow hood.
2. Set up a humidified incubator at 37°C with 5% (v/v) CO_2 for iPSC and kidney organoid culture.
3. See the key resources table for a complete list of materials.
4. Prepare solutions, Geltrex-coated 6-well plates and prewarmed culture media just before iPSC and kidney organoid culture following the recipes in the materials and equipment section.

Thawing iPSCs

Timing: 30 min

5. Remove the cryopreserved iPSC vial from the liquid nitrogen storage tank and thaw it in a 37°C water bath.
6. Add 3 mL of "iPSC maintenance medium" containing 1:100 revitacell into a 15-mL tube. Then, transfer the thawed cells into that tube in a dropwise manner.
7. Centrifuge the cell suspension at 300 rcf for 5 min at 18°C–24°C.
8. Discard the supernatant and resuspend the cell pellet in 2 mL "iPSC maintenance medium" containing 1:100 revitacell.
9. Add the cell suspension into one well of a pre-coated 6-well plate, shake the plate gently to distribute the cells homogeneously and place the plate into an incubator at 37°C, 5% CO_2.

Note: Each cryovial contains approximately 500,000–700,000 cells.

10. 24 h after iPSC thawing, refresh "iPSC maintenance medium" without revitacell.

Note: Proceed to cell passaging before the iPSC colonies become too large or reach 60%–80% confluency (approx. 3–5 days) (Figure 1A). For cell passaging, colony size is too large if big nearby colonies fuse (Figure 1B) and too small if they do not reach 50% confluency (Figure 1C). The growing speed of iPSCs varies by cell line and passage number.

iPSC maintenance culture – Passaging cells

Timing: 30 min

11. Aspirate culture medium, rinse the cells with 2 mL per well of prewarmed PBS.
12. Incubate the cells with 650 µL per well of EDTA 0.5 mM for 5–6 min at 18°C–24°C.

⚠ CRITICAL: Stop EDTA cell incubation when cells detach from the plate and form clumps visible by eye.

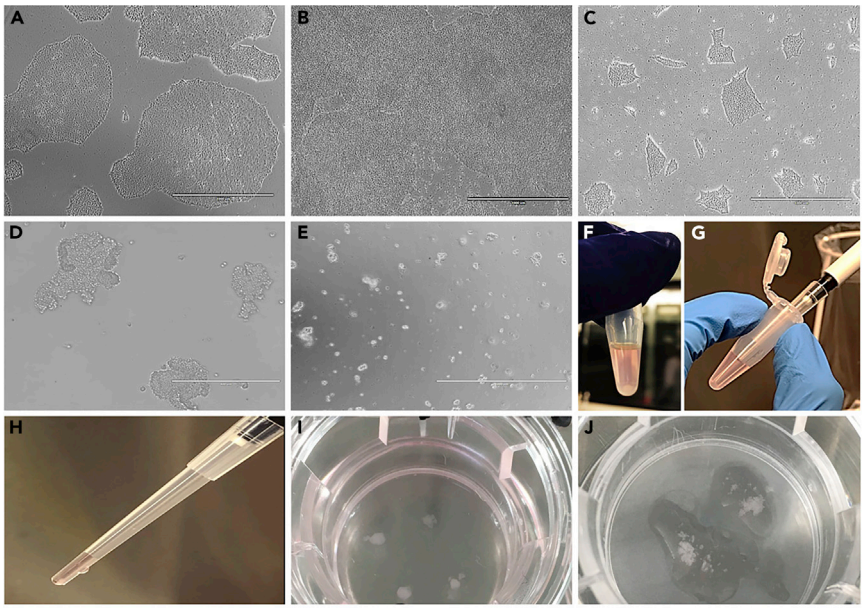

Figure 1. Overview of the iPSC maintenance culture, passaging and aggregation for making kidney organoids

(A) Representative bright field image of iPSC colonies with recommended size before cell passaging. Scale bar, 1,000 μm.
(B) Representative bright field image of iPSC colonies with bigger size than recommended before cell passaging. Scale bar, 1,000 μm.
(C) Representative bright field image of iPSC colonies with smaller size than recommended before cell passaging. Scale bar, 1,000 μm.
(D) Representative bright field image with required iPSC colony dissociation degree during cell passaging for iPSC maintenance culture. Scale bar, 400 μm.
(E) Representative bright field image with iPSC colony dissociation into single cells. Scale bar, 1,000 μm.
(F) iPSC pellet in a 1.5 mL tube prior to plating to generate cell aggregates which will derive into kidney organoids.
(G) Picking-up the iPSC pellet from (F) using a 200 μL wide-bore tip.
(H) Picked-up iPSC pellet from (F) in the bottom of a 200 μL wide-bore tip.
(I) Depositing of intact iPSC aggregates on a Transwell filter.
(J) Depositing of damaged iPSC aggregates on a Transwell filter.

13. Carefully aspirate EDTA using a 1 mL pipette, add 1 mL "iPSC maintenance medium" into each well and mechanically dissociate colonies by gently tapping against the plate.

⚠ CRITICAL: Do not break apart the colonies too much (a reduction to 25% of the colony size is recommended after this dissociation step) (Figure 1D). Do not pipette to avoid mechanical stress. Monitor dissociation under a bright-field microscope to ensure that cells are not dissociated into single cells (Figure 1E).

Note: Longer incubation time of iPSC colonies with EDTA will result in smaller colonies. To avoid cell damage, a longer incubation time than 7 min is not recommended.

14. Transfer the desired cell amount per well (recommended splitting ratio is about 1:4–1:8) into Geltrex-precoated 6-well plates containing 2 mL "iPSC maintenance medium" in each well.

Note: A 1:4 split from 80% confluency will lead to confluency in approximately 4 days in a well of the same size but it can vary between iPSCs cell lines from about 2 to 7 days.

15. Shake the plates back and forth and side to side to distribute the cells and keep them in the incubator until they reach 80% confluency to passage them again or to generate organoids. Refresh the culture medium daily between cell passages.

⚠ CRITICAL: For kidney organoid generation, iPSCs must have been passaged at least twice after thawing.

Note: Revise under a bright-field microscope that iPSCs maintain undifferentiated which means that they should display round colony morphology with high cell density and clear boundaries (Figure 1A) [troubleshooting 1]. Cell passaging can be continued until passage 50–60, then cells usually start to differentiate spontaneously.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Essential 8™ Flex Medium Kit	Thermo Fisher Scientific	A2858501
Essential 6™ Medium	Thermo Fisher Scientific	A1516401
Invitrogen UltraPure 0.5 M EDTA, pH 8.0	Thermo Fisher Scientific	15-575-020
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher Scientific	A1413302
Antibiotic-antimycotic (100×)	Thermo Fisher Scientific	15240062
CHIR99021	R&D Systems	4423/10
Recombinant Human FGF-9 Protein	R&D Systems	273-F9-025
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	H4784
Recombinant Human BMP-7 Protein	R&D Systems	354-BP-010
TrypLE Select Enzyme, no phenol red	Thermo Fisher	12563-029
RevitaCell Supplement	Thermo Fisher	A2644501
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300054
DMEM/F-12	Thermo Fisher Scientific	11320074
Fetal bovine serum	Merck	ES-009-B
Human epidermal growth factor (hEGF)	Sigma-Aldrich	E9644
Aldosterone, ≥95% (HPLC)	Sigma-Aldrich	A9477
[deamino-Cys1, D-Arg8]-Vasopressin acetate salt hydrate, ≥97% (HPLC)	Sigma-Aldrich	V1005
Recombinant Human/Feline CXCL12/SDF-1b	R&D Systems	351-FS-050
Accutase® solution, sterile-filtered, suitable for cell culture	Sigma-Aldrich	A6964
PBS pH 7.4	Gibco	10010056
Critical commercial assays		
Seahorse XFe96 FluxPaks mini	Agilent Technologies	102601-100
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies	103015-100
Seahorse XF Glycolysis Stress Test Kit	Agilent Technologies	103020-100
Seahorse XF DMEM assay medium pack, pH 7.4	Agilent Technologies	103680-100
Experimental models: Cell lines		
Human induced pluripotent stem cell line iPS134 (Cell passage number: 2–60)	SCTC Radboud UMC, The Netherlands	iPS134
Software and algorithms		
GraphPad Prism 9.3.1	GraphPad Software Inc.	https://www.graphpad.com/
Seahorse Wave Controller Software	Agilent Technologies	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Other		
Seahorse XF96 analyzer	Agilent Technologies	100900
Corning® Transwell® polyester membrane cell culture inserts, 24 mm Transwell with 0.4 μm pore polyester membrane insert, TC-treated, sterile	Sigma-Aldrich	CLS3450
Greiner Bio-One 6 Well Clear, Tissue Culture-Treated Multiple Well Plates with Lid	Greiner Bio-One	657 160
Scalpels, size 21, sterile	Omega Medical	16400122
Hemocytometer	Marienfeld	0640211

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Eppendorf™ Safe-Lock Tubes	Thermo Fisher Scientific	16439009
Centrifuge 5427 R	Eppendorf	5409000010
Falcon™ 15 mL Conical Centrifuge Tubes	Falcon	10773501
Falcon™ 50 mL Conical Centrifuge Tubes	Falcon	10788561

MATERIALS AND EQUIPMENT

- iPSC maintenance medium.

Reagent	Amount
Essential 8™ Flex Basal medium	48.75 mL
Essential 8™ Flex Supplement (50×)	1 mL
Antibiotic-Antimycotic (100×)	250 µL
Total	**50 mL**

Note: Make 50 mL aliquots of Essential 8™ Flex Basal medium and store them at −20°C for up to one year. This medium can be stored for max. two weeks at 4°C and needs to be warmed to 18°C–24°C before addition to the cells.

- iPSC differentiation media.

iPSC differentiation medium 1		
Reagent	Final concentration	Amount
Essential 6™ Medium	N/A	49.97 mL
CHIR99021 (10 mM)	6 µM	30 µL
Total		**50 mL**

iPSC differentiation medium 2		
Reagent	Final concentration	Amount
Essential 6™ Medium	N/A	49.85 mL
FGF-9 (1 mg/mL)	200 ng/mL	100 µL
Heparin (1 mg/mL)	1 µg/mL	50 µL
Total		**50 mL**

Note: Make 50 mL aliquots of Essential 6™ Flex Medium and store them at −20°C for up to one year. These media should be freshly prepared and need to be warmed to 18°C–24°C before addition to the cells.

- Kidney organoid medium.

Reagent	Final concentration	Amount
Essential 6™ Medium	N/A	49.942
BMP7 (100 µg/mL)	50 ng/mL	25 µL
hEGF (0.2 mg/mL)	10 ng/mL	2.5 µL
SDF-1b (50 µg/mL)	10 ng/mL	10 µL
Aldosterone 50 µM	10 nM	10 µL
Vasopressin 50 µM	10 nM	10 µL
Total		**50 mL**

Note: This medium should be freshly prepared and needs to be warmed to 18°C–24°C before addition to the cells.

Alternatives: STEMdiff™ APEL™ 2 Medium can be used alternatively to Essential 6™ Medium.

- Coating of cell culture plates.

2 mL per well of Geltrex™ 1:100 dissolved in DMEM/F-12 is used for 6-well plate coating.

Coated 6-well plates must be incubated for 1 h at 37°C before use to allow for matrix polymerization. Completely aspirate the Geltrex solution from the wells before seeding the cells.

⚠ CRITICAL: Once thawed, keep Geltrex™ on ice and use ice-cold tips and tubes for handling to avoid premature polymerization.

Note: Plates containing Geltrex 1:100 dissolved in DMEM/F-12 can be stored sealed with Parafilm® tape for max. one month at 4°C.

Alternatives: Matrigel™, Vitronectin XF™ or Laminin-521 can be used as an alternative to Geltrex™. Optimal cell plate coating can vary between iPSC lines.

- EDTA dissociation solution.

0.5 mM EDTA dissociation solution: Add 50 µL of 0.5 M EDTA stock into 50 mL of PBS (Ca^{2+}/Mg^{2+}-free).

Note: EDTA dissociation solution can be stored for six weeks at 4°C.

- Seahorse XF96 analyzer.

General manufacturer's instructions for performing a mitochondrial or glycolysis stress test can be respectively found at:

https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf.

https://www.agilent.com/cs/library/usermanuals/public/XF_Glycolysis_Stress_Test_Kit_User_Guide.pdf.

Alternatives: Our protocol is optimized for the Seahorse XF96 system (96 wells). Seahorse XF24 (24 wells) or Seahorse XF HS mini (8 wells) Analyzers could be alternatively used.

- Seahorse assay media.

Assay media are prepared with Seahorse XF DMEM pH 7.4 medium without phenol red supplemented with glucose, pyruvate and/or glutamine.

Mitochondrial stress test assay medium

Reagent	Final concentration	Amount
Glucose (1 M)	10 mM	200 µL
Pyruvate (100 mM)	1 mM	200 µL
Glutamine (200 mM)	2 mM	200 µL
Seahorse XF DMEM medium	N/A	19.4 mL
Total	**N/A**	**20 mL**

Glycolysis stress test assay medium		
Reagent	Final concentration	Amount
Glutamine (200 mM)	2 mM	200 µL
Seahorse XF DMEM medium	N/A	19.8 mL
Total	N/A	20 mL

Note: These media can be stored for 24 h at 4°C and need to be warmed to 18°C–24°C before addition to the cells.

Alternatives: Assay media can be prepared based on different types of media. For Agilent's recommendation on alternative media visit: https://www.agilent.com/cs/library/selectionguide/public/5991-7878EN.pdf.

- Preparation of stocks of substrates and inhibitors.

Stocks of substrates and inhibitors are prepared by dissolving the content of each tube of the Seahorse Stress Test Kits in the corresponding assay medium as depicted below.

Mitochondrial stress test assay reagent stock solutions			
Compound	Quantity per tube	Volume of assay medium	Stock concentration
Oligomycin	12.6 nmol	252 µL	50 µM
FCCP	14.4 nmol	720 µL	20 µM
Rotenone/Antimycin A	5.4 nmol (of both)	270 µL	20 µM

Glycolysis stress test assay reagent stock solutions			
Compound	Quantity per tube	Volume of assay medium	Stock concentration
Glucose	300 µmol	3,000 µL	100 mM
Oligomycin	72 nmol	1,440 µL	50 µM
2-DG	1500 µmol	1,500 µL	1 M

Note: These substrate and inhibitor stocks must be freshly prepared. Do not refreeze and reuse.

Alternatives: Substrates and inhibitors can be acquired individually and stored at −20°C as 100× stock solutions dissolved in DMSO (or H_2O in the case of glucose); which are used the day of the assay to prepare the volume of assay medium of stock solutions described in the tables above.

STEP-BY-STEP METHOD DETAILS

Making kidney organoids

Timing: 26 days

Timing: 8 days (for step 1a)

Timing: 18 days (for step 2a)

iPSC-derived kidney organoids were generated according to the protocol described by Jansen et al.,[5] an adapted version from the originally developed protocol for the generation of kidney organoids from human pluripotent stem cells.[4,10]

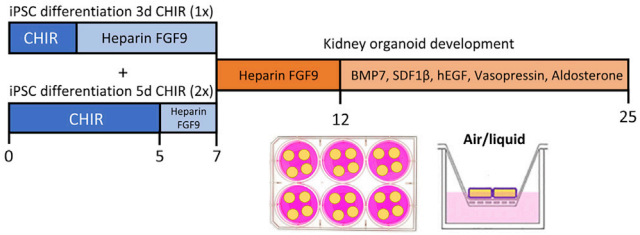

Figure 2. Schematic of the iPSC-derived kidney organoid culture protocol

After iPSC differentiation into either anterior intermediate mesoderm (AIM) (3d) or posterior intermediate mesoderm (PIM) (5d), cells are cultured in a 3D air-liquid interface Transwell system for an additional 18 days using a mixture of growth factors as indicated to stimulate morphogenic cues essential for nephrogenesis. BMP7, bone morphogenic protein 7; CHIR, CHIR 99021; FGF9, fibroblast growth factor 9; hEGF, human epidermal growth factor; SDF1β, Stromal cell-derived factor-1 β.

After iPSC differentiation into either anterior intermediate mesoderm (AIM) and posterior intermediate mesoderm (PIM) from primitive streak, cells are cultured in a 3D air-liquid interface Transwell system to stimulate morphogenic cues essential for segmented patterning of nephrogenesis. On this system, AIM cells will lead differentiation towards the ureteric bud which will give rise to collecting duct structures, while PIM cells will differentiate into the metanephric mesenchyme and, subsequently, into the nephron. Mature kidney organoids will be re-plated in Seahorse Cell Culture microplates 1 h prior to analysis (Figure 2).

1. iPSC differentiation into the ureteric bud and metanephric mesenchyme lineages:
   a. When iPSC colonies reach 60%–80% confluency in one well of a 6-well plate, aspirate the culture medium, rinse the cells with 2 mL per well of prewarmed (18°C–24°C) PBS three times and incubate the cells with 1 mL per well of prewarmed TrypLE Select solution for 2 min at 37°C.

   ⚠ CRITICAL: Check cell detachment efficiency by gently resuspending the cells with some of the TrypLE Select included in each well by using a P1000 pipette and monitor dissociation under a bright-field microscope to ensure that cells are dissociated into single cells. If cells do not detach into single cells, extend TrypLE incubation time at 37°C for an additional 30 s. To avoid cell damage, a longer incubation time than 3 min is not recommended.

   b. Add 3 mL "iPSC maintenance medium" to each well.

   Note: Do not pipette the cell suspension up and down more than ten times to avoid mechanical stress on the cells.

   c. Transfer the single-cell suspension to a 15-mL tube using a P1000 pipette.
   d. Add 2 mL of "iPSC maintenance medium" to each well to collect any leftover cells, transfer them to the same 15-mL tube and centrifuge the cell suspension at 300 rcf for 5 min.
   e. Remove the supernatant and resuspend the pellet in 2.5 mL of "iPSC maintenance medium" containing 1:100 revitacell.
   f. Determine the cell density using a hemocytometer.
   g. For iPSC134, seed 220,000 cells in 2.5 mL of "iPSC maintenance medium" containing 1:100 revitacell on Geltrex-coated 6-well plates.

   Note: The cell number for the seeding needs to be established for each individual cell line which will be in the range of 170,000–250,000 cells/well to reach a 100% cell confluency at day 7. As reference, by seeding a complete 6-well plate, approximately 180 kidney organoids will be obtained.

h. After 24 h (day 0), initiate iPSC differentiation by replacing the culture medium with 6 mL "iPSC differentiation medium 1".

Note: Revise under the microscope that cells are growing as single cells [troubleshooting 2].

i. At day 3, replace culture medium by 2.5 mL of "iPSC differentiation medium 2" in 1/3 of wells (3d cells), while in the rest replace it by 2.5 mL "iPSC differentiation medium 1" (5d cells).

Note: "3d cells" are referred to those treated with Essential 6™ medium supplemented with FGF9 and heparin in this step which induce iPSC differentiation into AIM, while "5d cells" to those treated with Essential 6™ medium containing CHIR 99021 which initiates differentiation to PIM. Cell medium does not need to be refreshed between day 0 and day 3.

j. At day 4, refresh the culture medium using 2.5 mL of the same type of medium than in the previous step in each well.
k. At day 5, 6 and 7 replace culture medium by 2.5 mL of "iPSC differentiation medium 2" in all the wells.

⚠ CRITICAL: Cell medium changing must be carried out very gently. Release cell culture medium drop by drop to avoid mechanical stress to the cells, which might lead to uncontrolled iPSC differentiation and cell detachment.

Note: Revise under the microscope that cells are growing as a monolayer and have reached 100% confluency. Importantly, due to the iPSC differentiation media not containing antibiotics, special attention must be paid on the sterile environment and signs of contamination [troubleshooting 3].

2. Cell 3D self-organization towards kidney organoids:
   a. Carefully, rinse the cells with 2 mL per well of prewarmed PBS three times.
   b. Incubate the cells with 1 mL per well of prewarmed Trypsin-EDTA (0.05%) for 2 min at 37°C.
   c. Add 3 mL DMEM/F-12 supplemented with 10% (v/v) FCS to each well.

Note: Do not pipette the cell suspension up and down to avoid mechanical stress on the cells.

   d. Transfer the 3d and 5d single-cell suspensions separately into different 50-mL tubes using a P1000 pipette.
   e. Fill each 50-mL centrifuge tube to 25 mL with DMEM/F-12 supplemented with 10% (v/v) FCS.
   f. Determine cell density of 3d and 5d live cells using a hemocytometer.
   g. Mix the 5d:3d suspensions based on their cell numbers in a 2:1 ratio and centrifuge the mixed 3d:5d cell suspension at 300 rcf for 5 min.
   h. Resuspend the cell suspension in Essential 6™ medium without cytokines in such a way that 200 μL cell suspension contain 200,000 cells.
   i. Make as many 200 μL aliquots of the previous cell suspension in 1.5 mL tubes as kidney organoids are needed.
   j. Centrifuge each tube 3 times at 300 rcf for 3 min at 18°C–24°C changing position by 180° per cycle to generate roundish cell aggregates.

Note: Centrifuging for 9 min only in one direction will lead to an elongated cell pellet which is not compact enough to be transferred onto the Transwell filter in one piece.

   k. Pick up pellets using 200 μL wide-bore tips and plate them on Transwell filters (4 pellets per filter) (Figures 1F–1I).

⚠ CRITICAL: To avoid damage of the aggregates (Figure 1J), use a P20 pipette set to 18 μL with 200 μL wide-bore tips to transfer aggregates. Gentle operation is required for this transfer process.

l. Add 1.2 mL Essential 6™ medium supplemented with 5 μM CHIR 99021 into the bottom part of each Transwell well to stimulate self-organizing nephrogenesis.
m. After 1 h, replace culture medium below the filter by "*iPSC differentiation medium 2*".

⚠ CRITICAL: Do not add cell culture medium on top of the filter. The air/liquid interface is crucial for the cells to stick together and develop into kidney organoids.

n. Refresh previous culture medium every two days.
o. On day 7 + 5, replace culture medium by kidney organoid medium (Essential 6™ medium supplemented with hEGF, BMP7, SDF1β, vasopressin and aldosterone).
p. Until d7+18, refresh previous culture medium every Monday, Wednesday and Friday.

⚠ CRITICAL: Avoid culture medium bubble formation below cell aggregates in the Transwell plates to ensure appropriate medium supply to kidney organoids (Figures 4A and 4B).

Note: The quality of kidney organoid formation can be evaluated by the identification of tubular structures under a bright field microscope. Further, it can be addressed by the analysis of specific marker genes at different stages of iPSC differentiation by qPCR or immunofluorescence, which is recommended when performing this protocol for the first times. Thus, LIM Homeobox 1 (LHX1), Empty Spiracles Homeobox 2 (EMX2) and GATA Binding Protein 3 (GATA3) are AIM markers, while Glial Cell Derived Neurotrophic Factor (GDNF), SIX Homeobox 2 (SIX2) and Homeobox A11 (HOXA11) are PIM markers, which can be studied at step 1_k. Mature kidney organoids show the presence of podocytes (Nephrin, NPHS1), proximal tubules (lotus tetragonolobus lectin, LTL), distal-like (E-cadherin, ECAD) and collecting duct-like (ECAD and binding protein 3 (GATA3)) cells [troubleshooting 3].[4,5,10,11]

Note: If treatments are needed to induce kidney organoid injury, they should be applied after completion of morphogenesis both on top and below the Transwell filter with 800 μL and 1.2 mL of E6 medium, respectively, to gain maximum exposure surface area. Treatments have to be maintained after re-plating into Seahorse microplates. Here, we show an example of kidney organoids treated with 2 ng/mL aristolochic acid (Sigma-Aldrich, A5512) for 72 h.

Sensor cartridge hydration

Timing: 12 h

Each probe tip of the sensor cartridge is spotted with a solid-state sensor material that detects changes in both pH and O_2 concentration. For correct functioning, they have to be hydrated in XF Calibrant for 12 h.

3. Lift the sensor cartridge from the utility plate and fill each well of the utility plate with 200 μL of XF Calibrant (Figure 3).

Optional: Sterile water can be used instead of XF Calibrant.

4. Place the sensor cartridge back on the utility plate, submerging the sensors in the XF Calibrant.
5. Place the assembled sensor cartridge and utility plate in a non-CO_2 37°C incubator for 12 h.

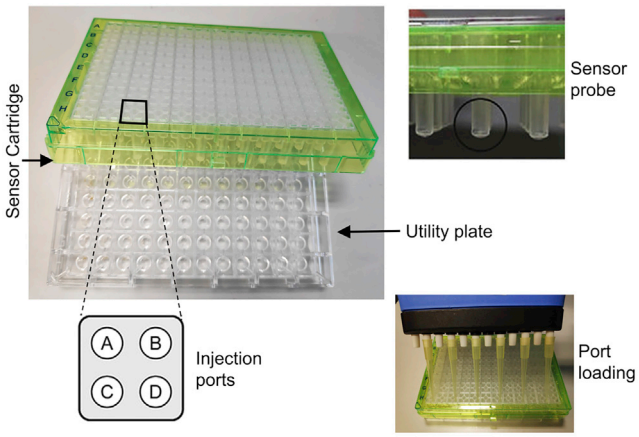

Figure 3. Seahorse sensor cartridge hydration and injection port loading

Seahorse sensor cartridge on top of a utility plate with injection ports A–D, with details of the O_2- and pH-sensitive probe tips of a Seahorse sensor cartridge and substrate/inhibitor stock solutions loading in the injection ports of the Seahorse sensor cartridge.

Note: To prevent evaporation of the XF Calibrant during this incubation time, seal the lid to the utility plate with Parafilm® tape or use a humidified incubator. Further incubation time does not affect negatively to the correct functioning of this device.

Re-plate organoids for Seahorse analysis

Timing: 1 h

Each kidney organoid must be plated in one well of a Seahorse XF96 cell culture microplate. Below, we describe step-by-step how to accurately re-plate individual organoids to guarantee reliable measurements.

⚠ CRITICAL: Before starting, prepare 20 mL of the corresponding Seahorse assay medium as indicated in the materials and equipment section and warm it to 37°C before using it.

6. Carefully wash organoids twice with 500 µL of the corresponding assay medium.
7. Remove the assay medium from the upper compartment of the wells of the Transwell plate. Place the insert upside-down on a flat surface and individualize every kidney organoid by cutting the porous membrane closely around each one by using a scalpel blade (Figures 4C and 4D).

Note: To minimize potential organoid drying, isolate all the kidney organoids within a well and proceed to next step.

8. Plate each kidney organoid in one well of a Seahorse XF96 cell culture microplate.
   a. Add 3 µL Geltrex™ (1:2 dissolved in DMEM/F-12) in the middle of the bottom of each well of a Seahorse XF96 cell culture microplate (Figures 4E and 4G).
   b. Immediately, place the organoid, including the Transwell membrane, in the middle of the well and in between the three nodes to adhere the organoid to the bottom of the plate (Figure 4F).

Note: Do not place kidney organoids in the A1, A12, H1 and H12 wells to keep them as background wells.

⚠ CRITICAL: Once thawed, keep Geltrex™ on ice and use ice-cold tips and tubes while manipulating to avoid early polymerization. For the same purpose, do not add Geltrex

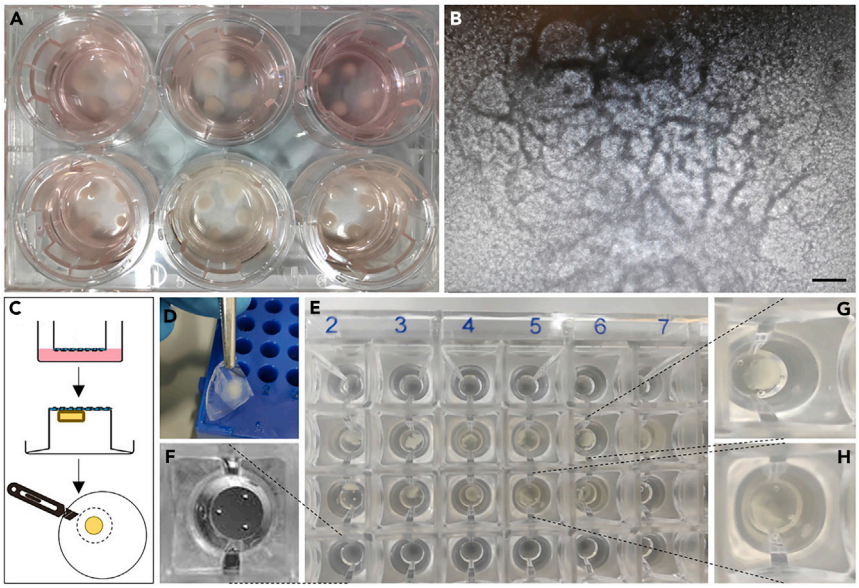

Figure 4. Overview of kidney organoid re-plating for Seahorse analysis

(A) iPSC-derived kidney organoids cultured in a 6-well Transwell plate on an air/liquid interface.

(B) Representative bright field image of a kidney organoid. Scale bar, 250 μm.

(C) Schematic depicting the strategy individualize every kidney organoid by cutting the porous membrane closely around each one by using a scalpel blade.

(D) Individualized kidney organoid by cutting the Transwell porous membrane.

(E) Re-plated kidney organoids on a Seahorse XF96 cell culture microplate.

(F) Empty well of a Seahorse XF96 cell culture microplate showing its three nodes at the bottom.

(G) Correct Geltrex™ immobilized kidney organoid in the middle of a well of a Seahorse microplate.

(H) Disrupted Geltrex™ immobilized kidney organoid in the middle of a well of a Seahorse microplate.

until you have isolated the organoid and it is ready to be placed into the well. Kidney organoids should not be embedded into the Geltrex™ matrix whose only function here is to get the membrane attached to immobilize the organoid on the bottom of the well. Wait 1 min before proceeding with the addition of the assay medium to allow Geltrex™ matrix polymerization [troubleshooting 4].

9. Add 180 μL of the corresponding assay medium into each well of the Seahorse XF96 cell culture microplate containing kidney organoids and in background wells [troubleshooting 5].

Optional: When analyzing the effect of drugs/treatment on bioenergetics, it might be necessary to maintain the treatment during the Seahorse assay. This needs to be decided based on the mechanism of action of the drug and the research purpose.

10. Place organoids in an incubator without CO_2 control at 37°C for 45 min.

Note: For an accurate assessment of the bioenergetic parameters, we recommend to have at least 5 wells per experimental condition.

XF mitochondrial stress test or glycolysis stress test

Timing: 3 h

Agilent has developed several assays to analyze different bioenergetic parameters. We describe an optimized assay medium composition, substrate and inhibitor concentrations and measurement

settings for the XF96 Analyzer to carry out mitochondrial and glycolysis stress tests in single kidney organoids.

11. During the incubation time of organoids in assay medium, prepare the stocks of substrate and inhibitor solutions as indicated in the materials and equipment section.
12. Substrate and inhibitor stock solutions should be loaded in the injection ports of the sensor cartridge. The device will inject the drugs in the order of port A to port D.
    a. For mitochondrial stress test:

Injection port	Drug	Stock concentration	Volume added to port	Final concentration in Seahorse plate
Port A	Oligomycin	50 μM	20 μL	5 μM
Port B	FCCP	20 μM	22 μL	2 μM
Port C	Rotenone/Antimycin A	20 μM	24 μL	2 μM

    b. For glycolysis stress test:

Injection port	Drug	Stock concentration	Volume added to port	Final concentration in Seahorse plate
Port A	Glucose	100 mM	20 μL	10 mM
Port B	Oligomycin	50 μM	22 μL	5 μM
Port C	2-DG	1 M	24 μL	100 mM

⚠ CRITICAL: Do NOT insert pipet tips fully into the injection ports to avoid the damage of probe sensors. A port guide provided with each XF96 Sensor Cartridge can be used to assist loading in the ports. If so, place the pipette tips vertically into the port loading guide holes. Otherwise, dispense the load allowing the pipette tips to touch port rims in the inside top part. The use of extended length pipette tips is also recommended.

13. Upon completion of injection port loading, place the sensor cartridge holder in the utility plate back into the incubator.

Optional: To analyze the metabolic response to an acute treatment, drugs can be injected during the mitochondrial or glycolysis stress test via the injection port A. In that case, move the injections corresponding to the mitochondrial or glycolysis stress test to port B, C and D, respectively and adjust concentrations of the inhibitors in injection ports according to the change in the volume resulting from adding preceding injection in port A.

14. Prepare the settings of the XF Analyzer.
    a. Open the Wave software.
    b. Open a mitochondrial or glycolysis stress test template and fill in the assay details. A detailed explanation about Wave software can be found in: https://www.agilent.com/en/product/cell-analysis/how-to-run-an-assay.
    c. Adjust the XF96 analysis settings in the "Instrument Protocol" section.
        i. For mitochondrial stress test:

Settings	Number of cycles	Action	Time
Basal	3 cycles	Mix	4 min
		Wait	2 min
		Measure	2 min
Oligomycin	3 cycles	Mix	4 min
		Wait	10 min
		Measure	2 min
FCCP	3 cycles	Mix	4 min
		Wait	2 min

(*Continued on next page*)

Continued

Settings	Number of cycles	Action	Time
		Measure	2 min
Rotenone/Antimycin A	3 cycles	Mix	4 min
		Wait	2 min
		Measure	2 min

ii. For glycolysis stress test:

Settings	Number of cycles	Action	Time
Basal	3 cycles	Mix	4 min
		Wait	2 min
		Measure	2 min
Glucose	3 cycles	Mix	4 min
		Wait	2 min
		Measure	2 min
Oligomycin	3 cycles	Mix	4 min
		Wait	10 min
		Measure	2 min
2-DG	3 cycles	Mix	4 min
		Wait	2 min
		Measure	2 min

d. Save your template.

15. Run the mitochondrial or glycolysis stress test [troubleshooting 6].
    a. Click on the "Run" button, the plate platform will be accessible and the Wave software will ask to insert the sensor cartridge in the XF96 Analyzer.
    b. Place the sensor cartridge holder in the utility plate in the XF96 Analyzer.

Note: The calibration step will take approximately 20 min.

c. After calibration, replace the utility plate by the Seahorse Cell Culture microplate containing the organoids and the XF96 analysis will automatically begin.

⚠ CRITICAL: Proceed immediately to organoid cell number determination after the experiment has finished.

Note: The analysis step will take approximately 2 h.

Organoid cell number determination

Timing: 30 min (for step 16)

Due to cell number variability among kidney organoids, determination of bioenergetic parameters requires normalization. Below we describe how to individually disaggregate kidney organoids and count their total cell number.

16. Carefully remove the assay medium from each well by using a pipette.

Note: Avoid aspiration to remove assay medium to minimize the risk of kidney organoid disruption or detachment.

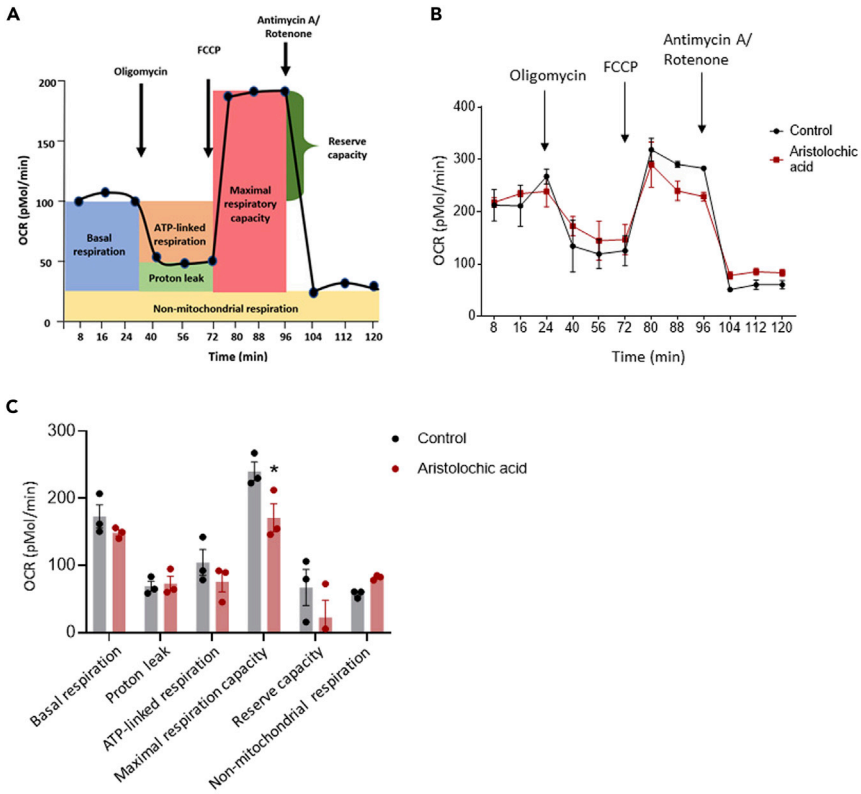

Figure 5. Mitochondrial stress test parameters in single kidney organoids

(A) Representative curve of OCR dynamics with commonly used reagents used for mitochondrial stress test.

(B) OCR associated to a mitochondrial stress test of kidney organoids treated with aristolochic acid (2 ng/mL) for 72 h.

(C) Bar chart showing mitochondrial respiration function parameters from (B). Data are normalized by the relative kidney organoid cell number. The difference between two independent groups was analyzed with the nonparametric Mann-Whitney test. Data are reported as mean ± standard error of mean (SEM), n = 3 biological replicates. A P-value of 0.05 or less was considered statistically significant. *p < 0.05 compared with the control condition. Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).

17. Add 100 μL Accutase per well and incubate the plate at 37°C for 10 min and disaggregate the kidney organoid by pipetting it up and down.

Note: In case of incomplete organoid disaggregation, prolong the incubation time (max. up to 20 min) at 37°C.

18. Add 100 μL DMEM/F12 medium containing 10% (v/v) FCS to each well to inactivate Accutase and mix by pipetting.
19. Count the number of cells using a hemocytometer. Calculate the number of cells in the total volume in each well which corresponds to the total number of cells in the respective kidney organoid.

Note: Alternatively, an automatic cell counter can be used to determine total cell number per organoid. Nuclear DNA content is also a reliable normalization method. Due to the presence of Geltrex™ matrix, normalization by protein content is not adequate. Organoid cell number should be included in the Seahorse Wave Desktop software for data normalization (for details see quantification and statistical analysis section).

EXPECTED OUTCOMES

The mitochondrial stress test is based on the oxygen consumption rate (OCR) data profile after sequentially adding modulators of mitochondrial function (Figure 5A). Basal respiration reflects

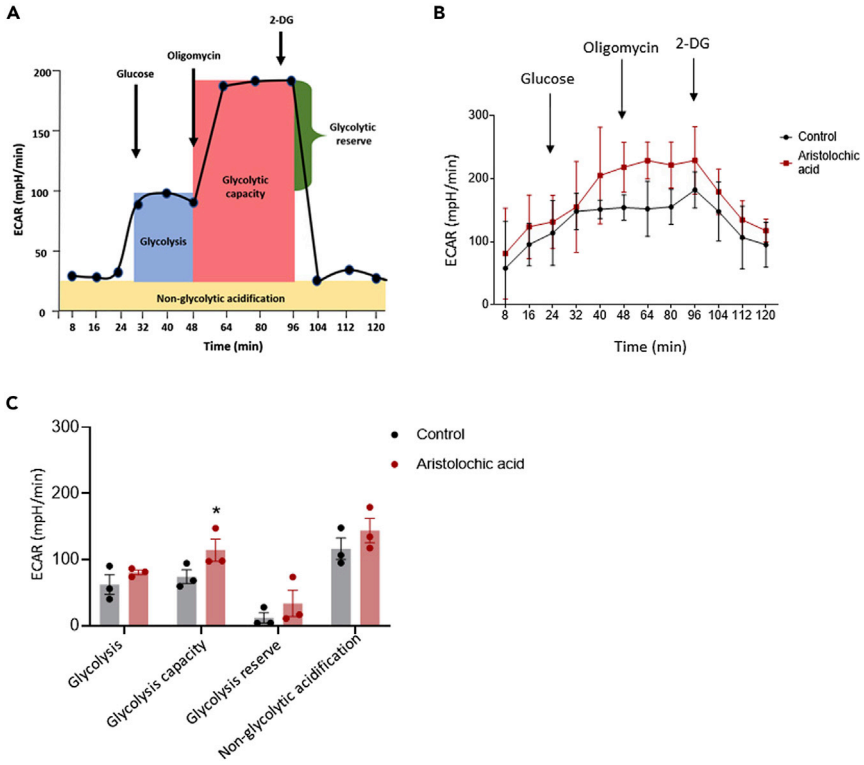

Figure 6. Glycolysis stress test parameters in single kidney organoids

(A) Representative curve of ECAR dynamics with commonly used reagents used for glycolysis stress test.

(B) ECAR associated to a glycolysis stress test of kidney organoids treated with aristolochic acid (2 ng/mL) for 72 h.

(C) Bar chart showing glycolysis function parameters from (B). Data are normalized by the relative kidney organoid cell number. The difference between two independent groups was analyzed with the nonparametric Mann-Whitney test. Data are reported as mean ± standard error of mean (SEM), n = 3 biological replicates. A P-value of 0.05 or less was considered statistically significant. *p < 0.05 compared with the control condition. Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).

the energetic demand of cells under basal conditions: the oxygen consumption of basal respiration used to meet both ATP synthesis and mitochondrial proton leak. ATP-linked respiration is reflected by the decrease in OCR following the injection of the ATP synthase inhibitor oligomycin, which is a portion of basal respiration. The remaining basal respiration not coupled to ATP synthesis after oligomycin injection shows the proton leak, which can be a sign of mitochondrial damage. Maximal respiration depicts the maximum capacity that the electron respiratory chain can achieve. It is measured by the injection of the uncoupler agent cyanide 4-(trifluoromethoxy) phenylhydrazone FCCP. Reserve capacity is the difference between maximal and basal respiration, which represents the capability of the cells to respond to an energetic demand. Non-mitochondrial respiration is the oxygen consumption due to cellular enzymes other than mitochondria and can be calculated after injection of the Complex I inhibitor rotenone and the complex III inhibitor antimycin A.[12,13]

The glycolysis stress test is based on the extracellular acidification rate (ECAR) data profile. It is the result of the net production and extrusion of protons into the extracellular medium, which reflects the conversion of glucose to pyruvate, and subsequently to lactate (Figure 6A). This assay measures basal glycolysis, glycolytic capacity upon blockage of mitochondrial ATP production using oligomycin which forces cells to increase glycolytic flux to meet ATP demand, and non-glycolytic extracellular acidification by blocking glycolysis the competitive hexokinase inhibitor 2-deoxyglucose (2-DG). Glycolytic reserve is the difference between glycolytic capacity and glycolysis rate, which reflects the capability of the cells to respond to an energetic demand as well as how close the glycolytic

function is to the cell's theoretical maximum. Non-glycolytic acidification is caused by cellular processes other than glycolysis and can be referred to the ECAR prior to glucose injection.

Here, we show both mitochondrial and glycolysis stress tests on kidney organoids under aristolochic acid-induced injury, which leads to a reduction in mitochondrial respiration and glycolysis, respectively (Figures 5 and 6). Figures 5B and 6B represent the rate data, which is the primary output of the Seahorse XF96 analyzer showing measurements of OCR and ECAR over time, respectively. The bar charts in Figures 5C and 6C represent the key parameters of mitochondrial and glycolysis function calculated as previously described.

QUANTIFICATION AND STATISTICAL ANALYSIS

After normalization, the mitochondrial and glycolysis stress tests can be analyzed by using the Seahorse Wave Desktop software (free to download from the Agilent website: https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897).

1. Open your experiment in the software.
2. Normalize your data for the organoid cell number.
3. Export data.
   a. For a mitochondrial stress test, export data as Seahorse XF Cell Mito Stress Test Report Generator.
   b. For a glycolysis stress test, export data as Seahorse XF Glycolysis Stress Test Report Generator.
   c. Raw data can also be exported to Excel or GraphPad Prism.
4. Analyze associated bioenergetic parameters, such as *basal respiration*, *maximal respiration*, *glycolysis*, and *glycolytic capacity* (Figures 5A and 6A).
5. To determine if observed differences are reproducible and biologically relevant, repeat the experiments at least three independent times [troubleshooting 7]. Statistical analysis can be applied to replicates, which shows the experimental accuracy.

LIMITATIONS

Limitations can be inherent to the organoid system or the Seahorse technology itself or derive from their combination. Differentiation efficiency, involving pluripotency, self-renewal and differentiation capabilities, depends substantially on the iPSC type and state, which associates with its genetic background, frozen batch, passage number and culture conditions. Organoids are heterogeneous as they consist of multiple cell types. Thus, bioenergetics analysis of organoids reflects the average metabolic phenotype of the different cell types that comprise the organoid. In some cases, modified differentiation protocols to enrich organoid cultures for a specific cell type have been described enabling its metabolic characterization.[14] In addition, due to the variability in the kidney organoid size and therefore in its cell number, bioenergetic parameter normalization of each kidney organoid to its corresponding total cell number is necessary to minimize the variability across the wells of the same experimental condition.

Our protocol applies Seahorse technology to mature kidney organoids which limits the analysis of the metabolic changes associated with the differentiation process. Efficiency of genetic manipulations carried out once kidney organoids have been generated can also be compromised. Therefore, when analyzing the metabolic consequences of gene editing, this efficiency should be considered in the interpretation of bioenergetic parameters. Re-plating organoids could potentially lead to cellular disaggregation. Long-term treatments can also be slightly interrupted by this fact. Re-plating must ensure that organoids maintain their original expanded disposition to avoid poor cell adhesion and inaccurate measurement of OCR or ECAR.

Mitochondrial and glycolysis stress tests cannot be directly applied to tissues, but they can measure respiration in isolated mitochondria, intact cells and organoids. Mitochondria do not receive the full dose of drugs in intact cells, as they might be impermeable to the mitochondrial complex inhibitors. Therefore, the permeability of the cell membrane and the complexity of cytoplasmic metabolism need to be considered when assessing the mitochondrial respiration in intact cells. Further, kidney organoid 3D self-organization limits the diffusion of mitochondrial drugs. It is also worth to consider that the bioenergetic parameters reported by Seahorse analysis are tightly coupled to the *in vitro* conditions, which involve the use of saturating concentrations of substrates and drug. This should be considered when extracting conclusions referring to *in vitro* culture conditions or *in vivo* analysis, where substrates can be present at different concentrations.[15]

TROUBLESHOOTING

Problem 1

Spontaneous iPSC differentiation or abnormal iPSC growth pattern (iPSC maintenance culture – passaging cells – step 15).

Potential solution

Roughly estimate the percentage of differentiated cells and monitor it over the next splitting event. If it is above 10%–15%, try to remove the differentiated cells by colony sinking. For that, transfer the medium containing the colonies (from *step 13*) into a 15-mL tube. Then, allow the colonies to sink by gravity. As it is expected that morphologically nondifferentiated and stable colonies are bigger, they will sink to the bottom of the tube, while differentiated single cells will stay in suspension the medium. After 10 min, aspirate the supernatant and resuspend the cell pellet in 1 mL "iPSC maintenance medium". Then, continue with *step 14*. If the problem persists, this cell culture needs to be discontinued and a fresh start with thawing new cells is recommended.

Problem 2

iPSCs are not growing as single cells just before differentiation induction (making kidney organoids – step 1_h).

Potential solution

Repeat iPSC cell seeding from a new iPSC maintenance culture. Try not to introduce air bubbles while pipetting. To improve cell dissociation into single cells, resuspend the cells with some of the TrypLE Select included in each well by using a P1000 pipette to dissolve bigger colonies and then use a P200 pipette to dissolve the remaining colonies with the narrower opening of the pipette tip.

Problem 3

Cells are not growing as a monolayer or are contaminated (making kidney organoids - steps 1_k and 2_p).

Potential solution

Repeat iPSC cell seeding and start a new iPSC differentiation round (step 1) from a new iPSC maintenance culture.

Problem 4

Kidney organoids do not stay immobile in the middle of the Seahorse microplate well after re-plating (re-plate organoids for Seahorse analysis - step 8_b).

Potential solution

Put the drop of Geltrex™ in the middle of a well of a Seahorse microplate and proceed very quickly to put the kidney organoid on it. Use a tip of a pipette to keep the organoid immobilized while Geltrex™ solidifies. Extend the time up to 2 min before adding the assay medium.

Problem 5

The organoids are disrupted or broken or their appearance indicates low mitochondrial fitness or viability in the Seahorse cell culture microplate (Figure 4H) (re-plate organoids for Seahorse analysis -step 9).

Potential solution

Minimize mechanical organoid shearing, avoid touching the kidney organoid surface or sharp movements, and increase slightly the diameter of the porous membrane around each organoid, which will allow easier manipulation by using a tweezer. A membrane piece with a maximum diameter of 5 mm is possible to be adjusted to the bottom of each well of a Seahorse XFe96 cell culture microplate (Figures 4C and 4D).

Problem 6

Minimal or unexpected changes in OCR after compounds injection (XF mitochondrial stress test or glycolysis stress test - step 15).

Potential solution

It is essential to control the stocks of the inhibitors and replace with fresh stocks when the organoid response to the drugs decreases over time. Also, check the organoids under the microscope before running the assay to make sure that the organoids are properly developed. Further, it should be checked whether the unresponsiveness of the organoids is a technical artifact or the actual phenotype of the organoids. For instance, when Oligomycin does not reduce OCR or only mildly increases ECAR, it suggests that Oligomycin is not functional. In this case, titration of drugs' concentration and exposure time is needed. Furthermore, it should be examined whether the setup of the experiments is optimal. For instance, it should be checked whether increasing glucose concentrations will enhance the glycolytic capacity, in order to check that glucose availability is not limited in the experiment.

Poor basal signal may be due to a long storage time of the Seahorse microplate well in a non-CO_2, 37°C incubator which could decrease the viability of organoid cells. Make sure the incubation time in a non-CO_2, 37°C incubator is at least 30 min but no more than 1 h.

Problem 7

High variability in OCR and ECAR measurement (quantification and statistical analysis - step 5).

Potential solution

Variability within technical replicates of an experiment can result from inaccurate plating due to Geltrex™ drops fusing to the wall of the plate instead of being centered in the well. Plating accuracy can be checked by microscope inspection after plating. Consider increasing the number of replicates and exclude "failed wells" from the analysis. To avoid variability between different experiments, make sure that the timing of the experiments is always similar.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rafael Kramann (rkramann@gmx.net).

Materials availability

This study did not generate new unique reagents, plasmids, or organoid lines.

Data and code availability

This study did not generate new datasets or codes.

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AUTHOR CONTRIBUTIONS

V.M., K.C.R., A.K.G., J.J., J.M., and L.M. developed the protocol. V.M. and R.K. wrote the manuscript, with revisions and comments from all the authors. Funding was provided by R.K.S and R.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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