Published in final edited form as:

Nat Protoc. ; 7(6): . doi:10.1038/nprot.2012.048.

Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells

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Abstract

Measurements of glycolysis and mitochondrial function are required to quantify energy metabolism in a wide variety of cellular contexts. In human pluripotent stem cells (hPSCs) and their differentiated progeny, this analysis can be challenging because of the unique cell properties, growth conditions and expense required to maintain these cell types. Here we provide protocols for analyzing energy metabolism in hPSCs and their early differentiated progenies that are generally applicable to mature cell types as well. Our approach has revealed distinct energy metabolism profiles used by hPSCs, differentiated cells, a variety of cancer cells and Rho-null cells. The protocols measure or estimate glycolysis on the basis of the extracellular acidification rate, and they measure or estimate oxidative phosphorylation on the basis of the oxygen consumption rate. Assays typically require 3 h after overnight sample preparation. Companion methods are also discussed and provided to aid researchers in developing more sophisticated experimental regimens for extended analyses of cellular bioenergetics.

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Note: Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS J.Z.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; E.N.: conception and design, data collection and analysis, manuscript writing; D.R.R.W.: collection and assembly of data, data analysis and interpretation; K.S., J.S.H., C.M.V.H., S.S.I., L.V.: technical support and assistance; C.S.M.: conception and design, data analysis and interpretation, manuscript writing; C.M.K.: conception and design, data analysis and interpretation, manuscript writing; M.A.T.: conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript and financial support.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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INTRODUCTION

Assessments of energy metabolism are essential for interrogating cell functions and also for the diagnosis and tracking of certain human diseases. Cells require energy in the form of ATP to support the biological processes of life, including growth, division, differentiation and many physiological activities. Studies in cellular energy metabolism encompass the biochemical pathways that generate and consume ATP, as well as carbon sources, signaling networks, intermediate metabolites and regulatory mechanisms that control these interconnected processes. Mitochondria, as the central organelle in a variety of essential cell functions including metabolism, have been the focus of many studies over many years. Protocols have been reported for studying the function of mitochondria isolated from yeast^{1,2}, from mouse tissues and cultured cells³, and from permeabilized fibers, tissues and cells⁴. However, measuring mitochondrial functions in hPSCs provides challenges not encountered in these other experimental systems.

In mammalian cell and developmental biology, considerable interest has emerged for understanding the functions and manipulating the activities of PSCs, especially those generated from human materials. hPSCs distinguish themselves from differentiated cells through the capacity to self-renew and thereby maintain the pluripotent state. With directed differentiation, hPSCs are at least theoretically capable of forming any of the 200+ cell types present in a child or adult, and therefore, beyond the innate curiosity associated with these cells, they hold tremendous potential for organ repair or replacement in the burgeoning field of regenerative medicine. One type of hPSC, human embryonic stem cells (hESCs), was first established by isolating and culturing cells obtained from the inner cell mass of human blastocysts over a decade ago⁵. Within the past 5 or so years, the exciting discovery of defined transcription factor reprogramming, and derivative techniques based on this process, has also lead to the generation of another type of hPSC, so-called induced pluripotent stem cells (iPSCs), from a variety of differentiated cell types⁶⁻⁹. Currently, both hESCs and human iPSCs (hereafter referred to as hPSCs) are routinely grown in Petri dishes, typically in small adherent clumps or colonies. Compared with most differentiated mammalian cell types, hPSCs also proliferate rapidly with shortened cell cycle times and a higher proportion of cells in S phase of the cell cycle^{10,11}. Given the enormous interest in these cells, much effort has been expended in unraveling the genetic and epigenetic control mechanisms of the self-renewing pluripotent state and many protocols have developed for early non-specific and lineage-specific directed differentiation with variable degrees of efficiency¹². However, much less attention has thus far been focused on studies of the unique energy and biosynthetic requirements either for maintaining the pluripotent state or for differentiation or reprogramming processes.

Development of the protocol

Recent studies have shown that PSC energy metabolism contrasts sharply with energy metabolism in most terminally differentiated cell types¹³⁻¹⁶. Glycolysis and oxidative phosphorylation (OXPHOS) are the two major mechanisms that mammalian cells use to produce ATP (Supplementary Fig. 1)¹⁷. It is well-known that most cancer cell types with intact mitochondria nevertheless convert to energetically less favorable glycolysis from OXPHOS in a metabolic transition termed the Warburg effect^{18,19}, facilitating a shift to anabolic pathways. Several adult stem cell types with functional mitochondria also have been reported to favor glycolysis over OXPHOS for energy production, in contrast to their differentiated progeny cells. For example, hematopoietic stem cells predominantly use glycolysis rather than OXPHOS, possibly because they reside in a hypoxic bone marrow niche, which limits available oxygen as a terminal electron acceptor^{20,21}. A similarly hypoxic microenvironment may also stimulate the glycolytic preference of tumor cell energy metabolism, at least in some pathologic settings. hPSCs with a relatively fast proliferation

rate also rely more heavily on glycolysis than OXPHOS for energy production, probably because of an associated increased flux through the pentose phosphate pathway, which is required to generate ribose and NADPH for nucleotide and lipid for biosynthesis to support rapid cell proliferation^{14–16}.

Unlike most mammalian cell lines, unique challenges for measuring mitochondrial metabolism in hPSCs include the growth of cells in small adherent clumps or colonies and the requirement for generally expensive and specialized culture medium that can practically limit the number of cells available for analyses. Therefore, to assess hPSC bioenergetics, we established an approach that keeps the mitochondria in their native intracellular environment, requires smaller samples than conventional methods of analysis, and minimizes manipulation of the experimental system to reduce material losses. Specifically, the extracellular acidification rate (ECAR), which approximates glycolytic activity under certain conditions, and the mitochondrial oxygen consumption rate (OCR), which is a key metric of mitochondrial function, can be determined simultaneously within the same small population of hPSCs using a commercially available XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Accurate XF24 measurements require the analyzed cells to be grown in a uniform monolayer configuration, which is not how hPSCs grow under standard culture conditions. This requirement must be met because the XF24 pH and oxygen sensors require a uniform detection environment, whereas nonuniform local cell densities result in nonreproducible error-prone measurements. To overcome this shortcoming, hPSCs and other cell types used in this protocol are treated with the Rho-associated kinase (ROCK) inhibitor, which maintains hPSCs as single cells after disaggregating hPSC colonies with retained viability and function; this is followed by plating in a homogenous monolayer before XF24 measurements²². Our protocol reproducibly assesses glycolytic and OXPHOS activities and can be used to establish basal and maximal respiratory capacities, evaluate mitochondrial uncoupling phenomena and can also be used to examine nonglucose carbon sources for bioenergetic studies in hPSCs and differentiated cell types¹⁴.

Comparison with other methods

Several protocols have been established for studying mitochondrial physiology and energetics including those that use purified mitochondria, detergent-permeabilized cells and intact cells. Because hPSCs are expensive to maintain and grow in small clumps at comparatively low cell numbers, these prior approaches have several shortcomings, which motivated development of the protocol we provide here.

Studies of mitochondrial metabolic function with detergent-permeabilized cells allow researchers to manipulate the experimental system but still keep mitochondria in their native intracellular environment⁴. Detergents such as digitonin and saponin, which have a high affinity for cholesterol²³, can be added to cells to selectively permeabilize the plasma membrane while leaving the outer and inner mitochondrial membranes intact. A crucial parameter for this approach is to titrate the appropriate detergent for the specific cells being used in order to establish an optimal concentration that opens the plasma membrane but not other organellar membranes. As a control, a mitochondrial enzyme such as matrix-localized citrate synthase is typically used to assess whether the mitochondrial membranes have been inadvertently opened²⁴. Permeabilization of the plasma membrane will cause the release of soluble cytosolic components, although this approach is still advantageous because mitochondria remain in contact with cytoskeletal and other native elements²⁵. Also, mitochondrial metabolites, such as succinate, glutamate and malate that cannot cross the plasma membrane in intact cells can now be added to mitochondria in permeabilized cells, facilitating OXPHOS studies of individual respiratory complexes. The permeabilized cell approach is also advantageous when cell numbers are limiting, because these studies can be performed with as few as 10^5 – 10^6 cells.

Metabolic studies with purified mitochondria provide the greatest flexibility but remove mitochondria from their native intracellular environment. Standard procedures for mitochondrial isolation based on differential centrifugation of cell or tissue homogenates have been reported previously^{3,26}. Isolated mitochondria are useful for a broad range of experiments including assays of the enzymatic function of individual respiratory chain complexes as well as the entire OXPHOS system, protein and RNA import studies, and studies on the transport of ions and metabolites across the mitochondrial membranes. However, this methodology has several disadvantages, particularly with regard to the purification of mitochondria from hPSCs. Mitochondrial isolation methods require extensive Dounce homogenization that can negatively affect the function of the mitochondria being collected. Typically, 30–40 Dounce strokes are required, and cell breakage can be difficult in cultured cells requiring even harsher conditions to achieve mitochondrial isolation. In addition, relatively large numbers of cells (e.g., $> 2 \times 10^8$ cells) are needed for optimal yield and quality³. For hPSCs, isolated mitochondria are frequently obtained uncoupled, regardless of whether they are uncoupled within cells or not, and the yield is characteristically low, mainly because the cytoplasm is scant and therefore the numbers of mitochondria per cell are fewer than in larger, differentiated cells with abundant cytoplasm, or than in cells specialized for OXPHOS, such as hepatocytes or cardiomyocytes. As a result, isolated hPSC mitochondria are often, but not always, unsuitable in quality or quantity for large-scale studies, such as traditional respiration measurements. However, when extensive biochemical analysis is not required, mitochondrial preparations from hPSCs can be useful for other crucial studies, such as the analysis of the assembly of respiratory complexes using blue-native gel electrophoresis with or without in-gel activity assays²⁷ and for mitochondrial transcription and translation studies. Researchers should be cautious, however, to gauge the extent of mitochondrial damage with isolation by using standard assays, such as citrate synthase activity or respiratory control ratios, whenever possible.

Methodologies for assessing mitochondrial function typically rely on respiration studies and other approaches. To facilitate this need, we developed an intact cell approach because this is currently the best method given the technical hurdles in working with small numbers of clumped hPSCs. This approach requires relatively few cells for assaying each condition of interest and the measurements are noninvasive and can be repeated multiple times in multiple assay wells. In addition, both glycolysis and respiration are measured simultaneously. The main disadvantage of this approach is that the method is limited to respiratory uncouplers and inhibitors (Table 1) or other reagents that readily cross an intact plasma membrane. Accordingly, mitochondrial metabolites such as glutamate, malate and succinate cannot be directly added to intact cells. Additional methods have also been developed for measuring glycolysis or OXPHOS *in vivo*, such as fluorescence- or luminescence-based methods that measure oxygen consumption (e.g., Becton Dickinson Biosensor plates)²⁸ and probes that quantify ECARs, such as a microphysiometer that measures acidification based on proton excretion²⁹. In contrast to the XF24 analyzer, however, a major disadvantage is that both of these later approaches cannot be done simultaneously on the same platform on the same small population of cells.

The traditional workhorse for measuring mitochondrial respiration has been the Clark-type oxygen electrode³⁰, which can be used for intact and permeabilized cells as well as on isolated mitochondria. The major benefit of the oxygen electrode is that a large number of inhibitors and substrates can be added with isolated mitochondria or permeabilized cells, so that the respiratory activity of the individual electron transport chain (ETC) complexes can be assessed in addition to the respiratory control ratio (RCR), which is an indication of coupling efficiency between substrate oxidation and phosphorylation, and the ADP/O ratio, which assesses the efficiency of mitochondrial OXPHOS³¹. The drawbacks of the oxygen

electrode are that large numbers of mitochondria or cells are typically required and that the chamber is stirred so cells cannot be adherent or in large clumps. Below we provide an XF24 analyzer protocol with glucose as the carbon source and directly compare this to measurements made with a conventional Clark-type oxygen electrode (Box 1; see also Tables 2 and 3) to highlight the utility of the Clark-type oxygen electrode for measuring all ETC complex activities in one run, even though a large number of cells are required. We also provide an XF24 analyzer protocol with a carbon source alternative to glucose, in this case free fatty acid (Box 2). All these protocols can be conveniently applied to multiple cell types as well. Figure 1 presents a flow chart of how these protocols relate if you are using hPSCs.

Experimental design

System optimization—The XF24 and associated experimental systems must be optimized for each cell type being assessed. Essential considerations at the beginning of an evaluation include whether or not the cells under study can be grown in large quantities at reasonable cost and effort and also what the physical growth characteristics of the cells are, such as adherent or suspension expansion and growth as single cells or clumps. For reproducible XF24 measurements, cells that grow in clumps require dispersion into monolayers without affecting their viability or function, which is the situation for hPSCs. Also, cells that grow in suspension need to be attached as a uniform monolayer to the bottom of an XF24 analyzer plate well, which can be the situation for white blood cells. In addition, a cell number titration to determine the optimal cell seeding density so that measurements are within the linear range of the XF24 analyzer is required. Titration of permeable substrates, such as respiratory uncouplers and inhibitors, also must be performed to find acceptable nontoxic dosing regimens over the time frame used for each experimental condition tested.

Applications of the method—OCR and ECAR measurements on the same small population of cells can be determined during culture in defined medium conditions. The ratio of OCR to ECAR can indicate cellular preference for OXPHOS versus glycolysis when mitochondria are coupled for oxygen consumption and energy generation through complex V (F_1F_0 ATP synthase) activity. Studies with a variety of cell types have indicated that ECAR values determined by the XF24 analyzer are a reliable measure of glycolytic rate, even though, in principle, extracellular acidification can be contributed by respiratory CO_2 and by monocarboxylates other than lactate^{32–36}. The addition or removal of nutrients in the culture medium can be informative for determining cellular energetic compensation or responses across extremes of abundance or starvation, especially when linked with additional assessments of cell growth, proliferation, division and death. Similarly, the use of respiratory inhibitors can be an informative method for interrogating mitochondrial functions in respiration and energy production. Judicious use of respiratory inhibitors singly or in combinations can indicate (i) mitochondrial and nonmitochondrial components of cellular oxygen consumption, (ii) oxygen consumed for ATP generation through the F_1F_0 ATP synthase versus oxygen consumed with passive proton leakage across the mitochondrial inner membrane, which reduces the mitochondrial membrane potential, and (iii) maximal respiratory capacity in coupled and completely uncoupled conditions. The utilization of alternative carbon fuels, such as free fatty acids, can be assessed by changing the cell growth medium to the carbon source of interest and repeating the same basic measurements using the XF24 analyzer and ancillary studies (Box 2). These basic measurements of cellular energy metabolism can be obtained in a single XF24 run of 2–3 h in duration, which requires appropriate repetition and controls to achieve statistical significance.

Controls

For each experimental plan, control XF24 analyzer wells are needed to establish background measurements. The control wells typically have no cells, but contain the same quantities and types of assay media and undergo the same agent injection procedures and measurements. Agent vehicle control injections, such as DMSO or ethanol, should also be included as separate assay plate wells to compensate for their influence, if any, in the experimental outcomes. For experiments measuring agent responses, basal level OCR or ECAR quantities can be used as no-treatment controls.

Summary

We provide the following protocols for researchers working on cellular energy metabolism mainly in the stem cell field but with concepts and approaches that generalize to studies of energy metabolism in all mammalian cell systems. This protocol serves as a starting point for those not familiar with mitochondrial manipulations and addresses how to measure basic glycolytic and OXPHOS metabolic pathway activities. Metabolism research is highly topical and represented with increasing frequency in all fields of mammalian cell biology, including topics related to PSC self-renewal and differentiation, reprogramming to pluripotency, mechanisms in metabolism, and basic and clinical cancer biology³⁷. As a researcher becomes more practiced, experiments can be refined to include permeabilized cells and isolated mitochondria.

MATERIALS

REAGENTS

Cell lines of interest—

- hPSC cell lines (hESCs: HSF1 and H1; hiPSCs: HIPS2 and HIPS18)⁸
- Normal human dermal fibroblast cells (NHDF; Lonza, cat. no. CC-2509)
- Rho0 143B TK⁻ (human osteosarcoma cells; a gift from D. Wallace, University of Pennsylvania)
- HEK293T cells (human embryonic kidney cells; ATCC, cat. no. CRL-11268)
- HeLa cells (human cervical cancer cells; ATCC, cat. no. CCL-2)
- MCF7 cells (human breast cancer cells; ATCC, cat. no. HTB-22)
- Ramos cells (human Burkitt lymphoma cells; ATCC, cat. no. CRL-1596)
- Raji cells (human Burkitt lymphoma cells; ATCC, cat. no. CCL-86)
- Nalm6 cells (human pre-B cell leukemia cells; DSMZ, cat. no. ACC-128)
- Mouse embryonic fibroblasts, mitomycin-C treated (MEFs; Millipore, cat. no. PMEF-CF)

Other reagents—

- Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific, cat. no. MT-10-013-CM)
- Mediatech Cellgro RPMI 1640 medium (Fisher Scientific, cat. no. MT-10-040-CM)
- Fetal bovine serum (FBS; Omega, cat. no. FB-01)

- MEM non-essential amino acids solution (NEAA, 100×; Invitrogen/Gibco, cat. no. 11140-050)
- L-Glutamine (100×; Invitrogen, cat. no. 25030-081)
- Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12; Invitrogen, cat. no. 11330-057)
- StemPro hESC SFM—human embryonic stem cell culture medium (StemPro medium; Invitrogen, cat. no. A1000701) includes: DMEM/F-12 with GlutaMAX (cat. no. 10565-018), StemPro hESC supplement (cat. no. A10006-01) and bovine serum albumin (BSA, cat. no. A10008-01)
- 2-Mercaptoethanol (Sigma, cat. no. M6250)
- Basic fibroblast growth factor (bFGF, R&D Systems, cat. no. 233-FB)
- ROCK inhibitor (Sigma, cat. no. H139)
- PBS (10×; Fisher Scientific, cat. no. MT-21-031-CV)
- Penicillin-streptomycin (100×; Fisher Scientific, cat. no. MT-30-002-CI)
- Trypan blue stain (Invitrogen/Gibco, cat. no. 15250-061)
- Trypsin-EDTA (0.5% (wt/vol), Invitrogen/Gibco, cat. no. 15400-054)
- Trypsin inhibitor, soybean (Invitrogen, cat. no. 17075-029)
- Dispase (5×; Stem Cell Technologies, cat. no. 7913)
- Matrigel (BD Biosciences, cat. no. 354277)
- XF calibrant solution (Seahorse Bioscience, cat. no. 100840-000)
- XF assay medium (Seahorse Bioscience, cat. no. 100965-000)
- Lysis buffer (see REAGENT SETUP)
- Bio-Rad protein assay dye reagent concentrate (5×; Bio-Rad Laboratories, cat. no. 500-0006)
- Poly-L-lysine (0.01% solution; Sigma, cat. no. P4832)
- Sodium palmitate (Sigma, cat. no. P9767)
- (+)-Etomoxir sodium salt hydrate (Sigma, cat. no. E1905)
- Lactate assay kit (Biovision, cat. no. K627-100)
- Ultra fatty acid-free BSA (Roche, cat. no. 03117405001)
- L-Carnitine (Sigma, cat. no. C0158)
- GlutaMax (100×, 200 mM; Invitrogen, cat. no. 35050)
- Sodium pyruvate (100 mM; Cellgro, cat. no. 25-000-CI)
- D-(+)-Glucose (Sigma, cat. no. G8270)
- All-*trans*-retinoic acid (Acros Organics, cat. no. 302-79-4)
- Digitonin (Fluka, cat. no. 37008-1G)
- Malic acid (malate; Sigma, cat. no. 240176)
- Adenosine 5'-diphosphate sodium salt (ADP; Sigma, cat. no. A2754)

- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma, cat. no. C2920)
- Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma, cat. no. C2759)
- Rotenone (Sigma, cat. no. R8875)
- Sodium succinate (Fisher Scientific, cat. no. S413-500)
- Malonic acid (malonate; Sigma, cat. no. M-1750)
- Potassium cyanide (KCN; Sigma, cat. no. 60178)
- Antimycin A (Sigma, cat. no. A8674)
- Oligomycin A (Sigma, cat. no. 75351)
- Sodium L-ascorbate (Sigma, cat. no. A7631)
- *N,N,N,N*-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; Sigma, cat. no. 87890)
- D-(+)-Sucrose (Fisher Scientific, cat. no. S2-212)
- EDTA (EMD, cat no. EX0539-5)
- KCl (Sigma, cat. no. P4504)
- NaCl (Sigma, cat. no. S3014)
- MgSO₄ (Sigma, cat. no. M2643)
- Na₂HPO₄ (Sigma, cat. no. S3397)
- NaH₂PO₄ (Sigma, cat. no. S8282)
- KH₂PO₄ (Fisher Scientific, cat. no. P285)
- MgCl₂ (Fisher Scientific, cat. no. M33)
- Tris base (Fisher Scientific, cat. no. BP152-10)
- Tris-HCl
- Ethanol
- DMSO
- Insulin

EQUIPMENT

Cell culture disposables—

- Petri dishes (BD Falcon)
- Centrifuge tubes (Eppendorf)
- Pipettes, pipette tips, filter units (Fisher Scientific)

Other equipment—

- XF24 Extracellular Flux Analyzer (for 24-well microplate assays; Seahorse Bioscience)
- XF96 Extracellular Flux Analyzer (for 96-well microplate assays (is also available); Seahorse Bioscience)

- XF24 FluxPaks (Seahorse Bioscience, cat. no. 100850-001)
- XF24 V7 cell culture microplate (XF24 assay microplate; Seahorse Bioscience, cat. no. 100777-004)
- Cell culture centrifuge (Beckman Coulter)
- Tissue culture hood (SterilGard)
- CO₂ incubator (set to 5% CO₂ and 37 °C; Sanyo)
- Non-CO₂ isotemp incubator (set to 37 °C; Fisher Scientific)
- Inverted microscope (with fluorescence, phase-contrast objectives; Olympus)
- Syringe (30 ml; BD, cat. no. 309650)
- Syringe-driven filter unit (0.22 µm or 0.45 µm, Millipore, cat. no. SLHV033NB)
- Countess automatic cell counter (Invitrogen)
- Bio-Rad Smartspec 3000 spectrometer (Bio-Rad Laboratories)
- Hansatech oxygen electrode (Hansatech Instruments)
- 701N 10-µl glass syringe (Hamilton, cat. no. HA-CH-80300)
- pH meter (SevenEasy InLab Routine Pro; Mettler Toledo)
- Falcon tubes
- Heated stir plates
- Tissue culture plates
- T75 tissue culture flasks
- Water bath
- Pasteur pipette
- Cell scraper

REAGENT SETUP

Stem cell medium—According to instructions provided with the StemPro hESC SFM kit, to 454 ml of DMEM/F-12 medium add 36 ml of BSA, 10 ml of supplement, 5 ml of 100× penicillin-streptomycin (optional), 3.6 µl of 14.3 M 2-mercaptoethanol (final concentration: 0.1 M). Store at 4 °C for up to 1 month.

DMEM or RPMI full medium—To 870 ml of DMEM or RPMI 1640 plain medium, add 100 ml of FBS, 10 ml of NEAA, 10 ml of L-glutamine and 10 ml of penicillin-streptomycin; mix well and store at 4 °C for up to 3 months.

bFGF solution (1,000×)—To reconstitute bFGF, dissolve the lyophilized powder with sterile 1× PBS to 10 µg ml⁻¹ and add BSA to a final concentration of 0.1% (vol/vol). It can be stored at – 80 °C for 3 months without detectable loss of activity.

Trypsin-EDTA (1×)—Dilute 10× trypsin (0.5% (wt/vol)) to 1× (0.05% (wt/vol)) in sterile 1× PBS. Store at – 20 °C in 5-ml aliquots. Thaw a small aliquot freshly every time; do not reuse.

Dispase solution—Dissolve 5× dispase (5 mg ml⁻¹) fivefold with DMEM/F-12 plain medium to a concentration of 1 mg ml⁻¹. Filter through a 0.22-μm filter unit. Prepare 1-ml aliquots and freeze them at – 20 °C for longer-term storage, or refrigerate them at 4 °C for up to 2 weeks.

Trypsin inhibitor—Dissolve nonsterile 50 mg of trypsin inhibitor powder into 50 ml of 1× PBS and vortex vigorously. After complete dissolution, filter the solution with a 0.22-μm filter unit. Preserve at 4 °C for up to 2 weeks.

ROCK inhibitor—Dissolve ROCK inhibitor in water to make a 10 mM stock solution. Prepare 10-μl aliquots and freeze at – 20 °C for up to 1 month.

Matrigel solution—Dilute Matrigel 60-fold into DMEM/F-12 plain medium before making Matrigel plates. A volume of 100 μl of Matrigel in 6 ml of DMEM/F12 is enough for one six-well plate.

XF assay medium—Add GlutaMAX to a final concentration of 2 mM. Add sodium pyruvate to a final concentration of 1 mM. Add glucose to a final concentration of 25 mM. (Note that these setups are assay dependent, especially when the assay is related to substrate utilization.) Freshly prepare this medium with each use.

Inhibitor preparations—Dissolve oligomycin A in ethanol to make a stock solution of 1 mM; dissolve FCCP in DMSO to make a stock solution of 1 mM; dissolve rotenone in DMSO to make a stock solution of 10 mM; dissolve antimycin A in ethanol to make a stock solution of 10 mM. Store all inhibitors at – 20 °C for up to 1 month. **! CAUTION** These agents are ETC inhibitors and can be acutely toxic. Wear gloves, protective clothing, face/eye shields and respiratory protection during preparation. Wash skin thoroughly after handling. Avoid release into the environment. **▲ CRITICAL** Inhibitors may be light sensitive; prepare them in dark tubes or store them in the dark.

KHB assay medium for fatty acid oxidation assays—Add 1.75 g of KCl, 32.45 g of NaCl, 1.2 g of MgSO₄ and 0.85 g of Na₂HPO₄ into 1 liter of water to make 5× KHB assay medium. Store at 4 °C for up to 3 months. On the day of the experiment, dilute 5× KHB assay medium with water to a 1× final concentration and add 4.2 mM MgCl₂, 0.4 mM glucose, 0.5 mM L-carnitine and 0.1 μM insulin (final concentration). Adjust the pH to 7.4 and filter sterilize.

Palmitate-BSA complex preparation—Make 1 mM sodium palmitate in 150 mM NaCl and 0.17 mM BSA in 150 mM NaCl. Heat the sodium palmitate solution to 70 °C on a heated stir plate until it becomes clear, and then add an equal volume of hot sodium palmitate to the BSA solution (the molar ratio between palmitate and BSA is 6:1). Stir at 37 °C for 1 h. Adjust the pH to 7.4. Aliquot into 1-ml glass vials and store at – 20 °C for up to 1 month.

Etomoxir stock solution—Dissolve (+)-etomoxir sodium salt hydrate in water to make a stock solution of 100 mM. Store the stock solution at 4 °C for up to 1 month.

Retinoic acid-induced differentiation medium—Dissolve retinoic acid into DMSO to make a 100 mM 10,000× stock solution, aliquot and store at – 20 °C for up to 3 months. To make the medium, add 1 μl of stock solution into 10 ml of StemPro medium without bFGF. Vortex to mix. Store at – 4 °C for no more than 2 weeks.

Respiration buffer for the Clark-type oxygen electrode—Prepare a 0.137 M NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 25 mM Tris-HCl solution with water; adjust the pH to 7.4 and filter sterilize. Store at −4 °C for up to 3 months.

Lysis buffer—This buffer is composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% (vol/vol) Triton X-100. Store it at −4 °C for up to 6 months.

PROCEDURE

Seed hPSCs onto the XF24 V7 cell culture microplate • TIMING 2 h (plus growing and incubation time)

1. Grow hPSCs in feeder-free conditions on Matrigel^{38–40} in a six-well tissue culture plate to ~80% confluence. See details for splitting and maintaining hPSCs on Matrigel plates in box 3. Alternatively, adherent differentiated cells, such as fibroblasts, can be grown in a six-well plate in appropriate medium (e.g., DMEM full medium) to ~80% confluence. For differentiated cells in suspension, such as Raji B lymphocytes, grow them in a T75 tissue culture flask in appropriate medium (e.g., RPMI full medium). Typically, exchange the medium for differentiated cells every other day or as often as needed to maintain culture growth.

▲ **CRITICAL STEP** hPSCs must be cultured in feeder-free conditions in order to exclude MEF feeder cells, which could alter assay outcomes considerably (see ANTICIPATED RESULTS). We therefore strongly recommend that hPSCs are grown in feeder-free conditions on Matrigel plates for a few passages to eliminate MEF contamination before seeding onto XF24 V7 cell culture microplates.

2. To collect hPSCs or adherent differentiated cells, aspirate the medium from the six-well plates and wash once with 1× PBS (pH 7.4) at room temperature (RT; 20 °C). Add 1 ml of 1× trypsin-EDTA and incubate for 7 min at 37 °C and then add 1 ml of trypsin inhibitor to stop the reaction. Transfer the cells into a 15-ml Falcon tube and spin them down at 200g for 5 min at RT, then wash once with 1× PBS (pH 7.4). To collect differentiated cells in suspension, spin down the cell suspension at 200g for 5 min at RT and wash once with 1× PBS (pH 7.4).

▲ **CRITICAL STEP** Before plating on Matrigel in XF24 V7 cell culture microplates, it is crucial to use trypsin to dissociate hPSCs into single cells to allow the formation of a uniform monolayer of cells at the bottom of the assay microplate. Methods for removing hPSCs with collagenase or dispase will leave the hPSCs in clumps, resulting in a heterogeneous layer of cells or colonies, which may cause inaccurate or inconsistent measurements of both OCR and ECAR.

3. Count the number of cells to obtain approximately 5×10^4 cells per well multiplied by the number of wells to be used per 24-well XF24 V7 cell culture microplate. Spin down the cells at 200g for 5 min at RT and resuspend each aliquot of 5×10^4 cells in 100 µl of stem cell medium supplemented with 10 µg ml^{−1} bFGF and 10 µM ROCK inhibitor. (For situations that require comparisons between hPSCs and differentiated cells, resuspend the differentiated cells in exactly the same medium as the hPSCs. If comparisons are not being made, use the appropriate medium for each type of differentiated cell being examined.) A total of 5×10^4 cells per well is an optimized number of hPSCs or differentiated cells, such as human fibroblasts, to provide reproducible readings within the linear range of the XF24 instrument detection system (see ANTICIPATED RESULTS).

▲ **CRITICAL STEP** ROCK inhibitor maintains the survival of hPSCs as single cells. In the presence of 10 µM ROCK inhibitor, appreciable cell death is not

observed within 1 d after cell collection and plating compared with collection and plating in the absence of ROCK inhibitor. ROCK inhibitor should be stored at -20°C in aliquots and used freshly every time. Thaw- and-freeze cycles will markedly decrease the activity of ROCK inhibitor. Titration of the number of cells placed into each assay well is also crucial for obtaining linear range measurements on the XF24 instrument. Cell number titrations should be performed for each new cell type examined.

4. Seed 100 μl of hPSCs or differentiated cells in suspension into each well of a 24-well XF24 V7 cell culture microplate precoated on the well bottoms with 100 μl of Matrigel DMEM/F-12 solution³⁸. When seeding, make sure to inject the cell suspension against the lower half of each well's wall, which is narrower than the upper part, so that the cells can settle down to the bottom of the well uniformly.
5. Incubate the seeded XF24 V7 cell culture microplate in a 5% CO_2 incubator at 37°C for at least 1 h to allow the cells to attach to the bottom of the wells. Then, gently layer another 100 μl of stem cell medium supplemented with 10 g ml^{-1} bFGF and $10\text{ }\mu\text{M}$ ROCK inhibitor onto the cells by placing the delivery tip against the upper wider wall in each well (this is the wider portion of the microplate wells that accommodates the XF24 sensors on the cartridge).

▲ **CRITICAL STEP** The two-step seeding procedure will allow the cells to disperse on the bottom of the wells uniformly and give the cells enough nutrients in the medium to grow overnight.

? TROUBLESHOOTING

6. Incubate the seeded XF24 microplate in a 5% CO_2 incubator at 37°C for ~16 h (overnight) to equilibrate the system before analysis. Proceed to Step 7 before the overnight incubation (i.e., do Steps 6 and 7 concurrently).
7. Open one XF24 FluxPak that contains two parts: a cartridge with O_2 and pH sensors and a 24-well microplate used for sensor hydration. Place 1 ml of XF Calibrant Solution into each well of the sensor hydration microplate and place the sensor cartridge onto the microplate. Ensure that all the sensors on the cartridge are immersed in the calibrant solution in the hydration microplate. Incubate the plate with immersed sensors in a non- CO_2 incubator at 37°C for ~16 h (overnight).

! **CAUTION** Do not hydrate the sensor-microplate combination in a typical tissue culture incubator set at 5% CO_2 (e.g., the incubator used for hPSC-seeded XF24 V7 cell culture microplates in Step 6), as the CO_2 will affect the pH in the calibrant solution and cause erroneous ECAR measurements.

Exchange assay medium and load the cartridge with injection solution • TIMING 1 h

8| After both plates have incubated overnight (on day 2), warm 50 ml of XF assay medium for each 24-well XF24 V7 cell culture microplate at 37°C . Adjust the pH to 7.4 at 37°C .

▲ **CRITICAL STEP** The pH is temperature dependent. To maintain the pH at 37°C during ECAR measurements with the XF24 instrument, the pH should be adjusted while the medium is in a 37°C water bath.

9| Aspirate the cell medium from each hPSC- or differentiated cell-containing XF24 V7 cell culture microplate without disturbing the cell monolayers attached on the well bottoms (put the suction tip against the middle of the lower half of the wall, and leave ~50 μl of medium in each well to avoid cell drying).

10| Gently wash each well once with 1 ml of XF assay medium at 37 °C. Then, calculate and add the volume of XF assay medium at 37 °C to each well to reach a final volume of 750 µl, which will depend upon the number of inhibitors or uncouplers that are subsequently injected into each well as described in Step 11, below. Return the plate into a non-CO₂ incubator at 37 °C until you are ready to load the plate onto the XF24 Extracellular Flux Analyzer.

11| Prepare 10× mitochondrial inhibitor or uncoupler injection solutions. For hPSC energy-profiling studies, prepare 10 µM oligomycin A by adding 20 µl of 1 mM oligomycin A stock solution into 2 ml of XF assay medium, 3 µM FCCP by adding 6 µl of 1 mM FCCP stock solution into 2 ml of XF assay medium, and 10 µM rotenone + 10 µM antimycin A by adding 2 µl of 10 mM rotenone and 10 mM antimycin A stock solutions into 2 ml of XF assay medium. Adjust each final solution to pH 7.4 and add 75 µl of each 10× injection solution into injection ports A, B or C, respectively. Return the hydration cartridge to the non-CO₂ incubator at 37 °C before setting up the analyzer run. After injection, the final additive concentrations are: 1 µM oligomycin, 0.3 µM FCCP, 1 µM rotenone and 1 µM antimycin.

! CAUTION Add the inhibitors directly to the XF assay medium to facilitate mixing and deliver it to cells as quickly as possible, as some inhibitors might stick to tube walls. Check the orientation of the cartridge, which is indicated by a notch on the lower left side of the cartridge. Check the order of injection ports. When injecting solutions, even if some wells are not used or set as background, add an equal volume of the injection solution or XF assay medium in the corresponding ports to establish correct well volumes for baseline (control) measurements. When working with a new cell type or line, the concentration of each inhibitor should be carefully titrated. Also, cell viability over the time of treatment needs to be determined, especially for long protocols with multiple time points.

Load the cartridge and the assay microplate and run the program • TIMING 3 h

12| Design a study protocol in the XF24 Extracellular Flux Analyzer software provided by the manufacturer. For analyzing hPSCs, an appropriate study protocol is to set the time to mix for 2 min, wait for 2 min and measure for 4 min.

A standard measurement flowchart with mitochondrial inhibitor or uncoupler additions for hPSC energy profiling is provided in Figure 2. It is noteworthy that additional variations or combinations of injection solutions are possible (see Fig. 3), depending on the experimental question(s) being addressed.

13| Hit 'START' and place the hydrated cartridge from the non-CO₂ incubator at 37 °C on top of a calibrant plate in the load position on the XF24 Extracellular Flux Analyzer.

14| Wait for the machine to calibrate the sensors. When the calibration is finished, the cartridge is kept on the machine while the calibrant plate in the load position is sent out. At this point, load the hPSC- or differentiated cell-containing XF24 V7 cell culture microplate taken from the non-CO₂ incubator at 37 °C into the load position and click 'CONTINUE'.

15| Unstimulated OCR and ECAR will be recorded first by the XF24 Extracellular Flux Analyzer. Ensure that both OCR and ECAR values are significantly above the background nonspecific control well values. The control well(s) should have no cells but contain the same amounts and types of assay medium and injection solutions. For example, OCR should be above 10 pmol min⁻¹ and ECAR above 10 mpH min⁻¹. After solution injections from ports A, B and C, OCR responses to oligomycin A, FCCP and rotenone + antimycin A will be recorded to determine the cellular bioenergetic profile.

? TROUBLESHOOTING

16| After the XF24 Extracellular Flux Analyzer run is finished, remove the assayed XF24 V7 cell culture microplate and place it in a 37 °C incubator for protein concentration determination, and then hit 'CONTINUE' to end the program.

Determination of protein concentration and data analysis • TIMING 1 h

17| Aspirate off the XF assay medium from each well of the analyzed microplate without disturbing the hPSC or differentiated cell monolayer attached at the bottom of each well. Save the aspirated XF assay medium from each well separately for lactate determination assays. Wash each well once with 1 ml of 1× PBS (pH 7.4) at RT.

▲ **CRITICAL STEP** Check the hPSC monolayer with a phase-contrast light microscope to determine whether cells remained attached at the bottom of the plate and appear viable. Some inhibitors/uncouplers, such as antimycin A, may kill the cells, especially when co-incubated for an extended period of time. Also, some non-hPSC cell types, such as HEK293T cells, are more loosely attached than others and liquid aspiration can cause detachment and loss of material. In these cases, the protein concentration will be underestimated and OCR or ECAR values per unit of protein will be overestimated.

A poly-L-lysine plate coating can be attempted to overcome poor cell attachment in these cases.

18| Add 30 µl of lysis buffer to each well and incubate the plate on ice for 30 min.

19| Remove 10 µl of the cell lysate, add it to 1 ml 1× Bio-Rad protein assay dye reagent, mix well and measure the protein concentration of samples with an appropriate spectrometer (we use a Bio-Rad Smartspec 3000 spectrometer).

? TROUBLESHOOTING

20| Report the OCR and ECAR values. They can be reported as absolute values (pmol O₂ consumed per min for OCR and mpH change per min for ECAR), either on a per-cell basis or on a per-unit of protein basis. When using a per-cell basis, the data can be normalized to number of cells seeded on the day before the assay, assuming there is no significant difference in the proliferation rate for the entire cell types used.

Alternatively, the number of cells can be counted right after the assay or extra wells can be seeded with the same number of cells that can be counted at the time the assay is done. For energetic profiling experiments, such as those measuring responses to oligomycin A, FCCP and rotenone + antimycin A, OCR can be analyzed as the percentage change relative to the unstimulated OCR following inhibitor/uncoupler injections. Take the average of at least three replicate wells and calculate the standard deviation of the values obtained for the samples or calculate the standard error of the mean. Replicate experiments on different days should be run to assess run-to-run or systematic variability.

? TROUBLESHOOTING

21| Determine the lactate content of the aspirated XF assay medium from each well with the lactate assay kit according to the manufacturer's protocol. To do this, add 50 µl of XF assay medium and lactate standard samples into a reaction mix containing lactate dehydrogenase in a 96-well plate. Lactate is oxidized by lactate dehydrogenase to generate a product that interacts with a probe to produce a color change (max = 450 nm) recorded by a spectrometer. Plot optical density values at 450 nm (OD₄₅₀) values against nanomoles of lactate for each of the lactate standards. Fit the OD₄₅₀ of the XF assay medium onto a standard curve to calculate the lactate content. The level of accumulated lactate in the medium should be approximately proportional to the ECAR

measured by the XF24 Extracellular Flux Analyzer, assuming the lactate level in the starting XF assay medium is the same.

! CAUTION A pH change in the XF assay medium results from two main factors. The first is from the production of lactate via glycolysis and the second is CO₂ produced from the tricarboxylic acid (TCA) cycle that is dissolved in the XF assay medium. Typically, lactate produced from glycolysis is the dominant contributing factor to the pH change of the XF assay medium. However, it is important to compare ECAR with the lactate level in the medium to confirm that the ECAR is indeed an indicator of glycolysis.

? TROUBLESHOOTING

Troubleshooting advice is provided in Table 4.

• TIMING

Here we summarize the experimental procedures and the time needed for each step (Fig. 1).

Step 1, growing sufficient numbers of hPSCs on feeder-free Matrigel plates: ~ 4–5 d

Steps 2–5, seeding hPSCs in a XF24 V7 cell culture microplate: 2 h

Steps 6 and 7, incubating hPSCs at 37 °C in a CO₂ incubator and hydrating a sensor cartridge at 37 °C in a non-CO₂ incubator: 16 h (overnight)

Steps 8–11, exchanging XF24 assay medium and loading the sensor cartridge with injection solutions: 1 h

Steps 12–16, loading the sensor cartridge and assay microplate and running the XF24 Extracellular Flux Analyzer detection program: 3 h

Steps 17–21, determining protein and lactate concentrations and collecting data for analyses: 1 h

Box 1, measuring oxygen consumption with a Clark-type oxygen electrode: 3 h

Box 2, measuring extracellular flux with free fatty acid as the carbon source: 3 h

Box 3, maintenance and splitting of hPSCs on Matrigel plates: 1 h

ANTICIPATED RESULTS

For each type of cell, a prerequisite control experiment is the titration of the number of cells seeded into XF24 V7 cell culture microplates. When 20,000–75,000 hPSCs or human fibroblasts (NHDFs) are seeded, OCR values are within the linear detection range of the instrument; however, ECAR values for 75,000 human fibroblasts become inaccurate and plateau (Fig. 4a). On the basis of our analysis, a range of 20,000–50,000 hPSCs or human fibroblasts provides robust detection signals over the linear range of the instrument for both OCR and ECAR measurements. When there are less cells available, a XF96 analyzer with a 96-well assay plate can be used to scale down to the fewer number of cells. In that event, careful adjustment of the mixing times before each measurement is required to ensure sufficient oxygen equilibration in a smaller assay volume. When the number of cells for different cell types can be kept in the same instrument linear range, this facilitates direct OCR and ECAR comparisons between different cell types. With a constant number of cells, either the absolute OCR or ECAR levels, normalized to protein concentration, or the ratio of

OCR/ECAR can be graphically plotted. Figure 4b shows OCR/ECAR ratios for two hESCs (HSF1 and H1), two hiPSCs (HIPS2 and HIPS18) and human fibroblasts (NHDFs). Notably, the OCR/ECAR ratio is typically not affected with or without the addition of ROCK inhibitor, as shown for NHDFs (Supplementary Fig. 2). Low OCR/ECAR ratios for the hPSCs indicate their relatively higher reliance on glycolysis compared with fibroblasts¹⁴. For all experiments presented herein, data are presented as the mean $\pm$ s.d. A two-tailed test was used with $P < 0.05$ considered statistically significant. Using this method of analysis, we have also recently reported a progressive shift from a low to a high OCR/ECAR ratio during the process of hPSC differentiation¹⁴.

In addition to determining basal OCR and ECAR measurements, a key protocol capability is to analyze the hPSC bioenergetic profile with a series of mitochondria-specific inhibitors or respiration uncouplers. For example, oligomycin A inhibits the mitochondrial F_1F_0 ATP synthase, FCCP is a mitochondrial proton ionophore (uncoupler) and rotenone and antimycin A are ETC complex I and complex III inhibitors, respectively (Fig. 5a). Because they are membrane permeable, these inhibitors/uncouplers work well with intact cell studies in the XF24 Extracellular Flux Analyzer. To identify an OCR value at which coupled respiration is inhibited, cells are exposed to 1 μ M oligomycin A to prevent proton movement through the F_1F_0 ATP synthase, which has the effect of increasing the proton gradient across the mitochondrial inner membrane. The addition of 0.3 μ M FCCP re-establishes proton movement nonspecifically across the mitochondrial inner membrane and results in complete uncoupling of electron transport from ATP generation by the F_1F_0 ATP synthase, yielding an OCR value for maximal O_2 consumption for the cell type being examined. Subsequent addition of rotenone + antimycin A blocks proton pumping by the ETC and rapidly cripples mitochondrial O_2 consumption completely. This sequence of added inhibitors/uncouplers provides the following energy-profiling data: (i) the maximal respiration capacity, which is the difference between FCCP-induced OCR and rotenone + antimycin A-blocked OCR; (ii) the component of OCR used to generate ATP, which is the difference between the basal and oligomycin A-repressed OCR; and (iii) the component of OCR representing passive proton leakage across the mitochondrial inner membrane, which is the difference between oligomycin A and rotenone + antimycin-A inhibited OCR (Fig. 5a). The OCR 'drift' over time after oligomycin addition for HSF1 cells and after FCCP addition for NHDF cells is not due to changes in apoptosis or cell cycle parameters¹⁴, has also been reported for studies in additional cellular contexts by other investigators^{41,42}, and may reflect distinct drug response kinetics in different cell types. Notably, our sample data show that hPSCs (HSF1 cells) consume O_2 at maximal capacity in the basal state and that only 60–70% of O_2 consumption is used to generate ATP (Fig. 5b). By contrast, human fibroblasts (NHDFs) respire at about half of their maximal capacity in the basal state, and almost all O_2 consumed is used to synthesize ATP in the mitochondrial F_1F_0 ATP synthase (Fig. 5b). These results are reproducible with additional hPSCs and with double the concentration of pyruvate in the growth medium, which eliminates the concern that limited carbon substrate is responsible for a reduced maximal respiratory capacity¹⁴.

The XF24 Extracellular Flux Analyzer versus conventional approaches

To illustrate that the XF24 Analyzer can generate comparable results as the conventional method, we performed the two methods in parallel. The measured OCR for basal-state, intact hPSCs (HSF1) using the XF24 Analyzer is 73.40 ± 8.57 pmol min⁻¹ per 5×10^4 cells (Fig. 6a)¹⁴. The OCR measured for basal-state, intact HSF1 cells from the slope of the polarographic tracing using a Clark-type oxygen electrode (after 'add cells' at 2 min 44 s and before 'digitonin' permeabilization at 4 min 21 s in Fig. 6b) is 7.95 ± 1.40 nmol min⁻¹ per 5×10^6 cells¹⁴. From these previously published data, the calculated OCR per individual

HSF1 cell is similar between the two methods, although the conventional oxygen electrode measurements require 100-fold more cells to obtain reproducible results (Fig. 6a).

Despite the XF24 Extracellular Flux Analyzer's requirement for fewer cells than a Clark-type oxygen electrode, the electrode approach maintains an advantage by being able to determine the activities of individual ETC complexes in one experimental run. This is because permeabilized cells are continuously stirred in suspension with an electrode system, and manual injections with a Hamilton syringe theoretically permit an unlimited number of substrate and inhibitor deliveries until all of the oxygen in the assay buffer is consumed. This advantage is demonstrated in a polarographic tracing that determined the individual ETC complex activities for hPSCs (HSF1; Fig. 6b). The slope between the addition of a substrate and an inhibitor of the ETC complex being examined indicates the activity of that complex. For example, the slope between succinate (substrate) and malonate (inhibitor) additions indicates ETC complex II activity. As anticipated from the overall oxygen consumption data, ETC complexes I to IV in HSF1 cells are functional, as previously reported¹⁴. For ECAR, the difference between basal-state HSF1 cells and retinoic acid-induced differentiated progeny cells is an approximately 75% reduction when measured using the XF24 Extracellular Flux Analyzer and an approximately 80% reduction when measured using a commercial lactate assay kit (Fig. 6c). Both results support a similarly sized shift from glycolysis to respiration during hPSC differentiation, as reported previously^{14,16}.

Fatty acid oxidation measurements for hPSCs and differentiated cells

Cancer cells and mouse ESCs use fatty acid oxidation for ATP production, especially when these cell types are under metabolic stress, such as in conditions of glucose deprivation or hypoxia⁴³. Our protocol can be modified to interrogate alternative carbon sources, such as free fatty acids, as energy sources in hPSCs and their differentiated progeny. In Figure 7a, the exogenous free fatty acid palmitate, which can be internalized by intact cells, was injected into the XF24 assay medium, followed by the addition of etomoxir and detection with the XF24 Extracellular Flux Analyzer. The component of OCR attributed to palmitate oxidation is determined by the addition of etomoxir, which freely diffuses into cells and is an inhibitor of carnitine palmitoyltransferase 1 (CPT1), an enzyme that transports long-chain fatty acids across the mitochondrial inner membrane, thereby effectively inhibiting fatty acid oxidation. When 200 μ M palmitate is added to human fibroblasts (NHDFs) over 30 min, the OCR level increased by ~20%, which was subsequently inhibited by the addition of 100 μ M etomoxir (Fig. 7a). These data support the increase in OCR as being specifically from the oxidation of the added free-fatty acid palmitate (Fig. 7a). By contrast, palmitate addition does not increase the OCR in hPSCs (H1), suggesting that hPSCs do not use exogenous fatty acids as a carbon source for OXPHOS (Fig. 7a). Similar results were obtained with HSF1 cells using a dose escalation of added palmitate. The OCR increase in HSF1 cells is < 5%, whereas the OCR increase in human fibroblasts (NHDFs) is proportional to the added palmitate and can be as high as ~30% (Fig. 7b). The data also show that the OCR in HSF1 cells cannot be increased by increasing doses of added palmitate.

Using the XF24 Extracellular Flux Analyzer with other mammalian cell types

This energy-profiling protocol can be used for hPSCs and additional mammalian cell types, including those that grow in suspension, such as human blood lineage cells. In Figure 8, a wide range of cell types was profiled for OCR/ECAR ratios and absolute OCR and ECAR values. Rho0 143B TK⁻ is a human osteosarcoma cell line depleted of its mitochondrial DNA. Therefore, this line cannot respire and, as anticipated, these cells have the lowest OCR and high ECAR activities because they rely exclusively on glycolysis for energy production. hPSCs (HSF1) are also highly glycolytic, even though hPSCs do respire at their

maximal capacity (Figs. 5a and 8a)¹⁴. HEK293T and HeLa cancer cells have OCR/ECAR ratios that show robust glycolytic and respiratory activity, which is perhaps an indication of high metabolic activity to support a high rate of cell proliferation. Of note, MCF7 human breast cancer cells appear to respire well, with an OCR > 200 pmol min⁻¹ per 50,000 cells. This occurrence might not be anticipated from the Warburg effect in cancer cells^{18–19,44}, although recent studies show that different cancer cell types or lines of the same type of cancer do not all have the same metabolic profile and some cancers are more oxidative than glycolytic^{45,46}. The Ramos, Raji and Nalm6 lines, representing B lymphocyte lineage cancers, all show absolute OCR and ECAR values that are much lower compared with other cell types. The size of these cancer B cells (<10 µm in diameter) is much smaller than that of other cells in the comparison (~20–30 µm in diameter), suggesting that cell size and volume may be important considerations because of varying protein/mitochondrial content per cell when comparing the absolute level of OCR or ECAR across different cell types¹⁴. Last, mitomycin-C-treated MEFs show the highest respiratory activity among all cell types examined. Their OCR is ~4.5-fold higher than HSF1 cells, whereas their ECAR is about half that of HSF1 cells. This large difference in energy profiles strengthens the importance of preparing hPSCs under feeder-free conditions to avoid MEF contamination and inaccurate OCR and ECAR measurements. On the basis of our data, even a contamination of 5% MEFs in a XF24 study on hPSCs could result in an ~17.5% overestimation of OCR and an ~20% overestimation of the OCR/ECAR ratio. Thus, growing hPSCs in feeder-free conditions is important for eliminating this potential error in energy-profiling measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Tang for hESCs. Supported by CIRM grants RS1-00313, RB1-01397, TB1-01183, TG2-01169, a training grant from the Broad Stem Cell Research Center at the University of California Los Angeles, and US National Institutes of Health grants GM061721, GM073981, PNEY018228, P01GM081621, CA156674 and CA90571. C.M.K. is an Established Investigator of the American Heart Association and M.A.T. was a Scholar of the Leukemia and Lymphoma Society. We thank D. Wallace (University of Pennsylvania) for Rho0 143B TK⁻ human osteosarcoma cells.

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Box 1**Measuring oxygen consumption with a Clark-type oxygen electrode •
TIMING 3 h**

The assembly, calibration and maintenance of the oxygen-sensing electrode and the operation of the Oxygraph software for the electrode have been described previously³ and researchers should use the instruction manual provided by the manufacturer (Hansatech Instruments is an affordable example that we have illustrated). Here we present an optimized protocol for assaying hPSC oxygen consumption using a traditional Clark-type oxygen electrode for bioenergetic profiling. As explained previously, experiments with a Clark-type oxygen electrode are challenging to perform because the number of hPSCs is often a limiting factor. In theory, the same approach with inhibitors or uncouplers as described with the XF24 analyzer could be used, but the oxygen electrode can also be advantageous for measuring the activities of individual respiratory complexes using complex-specific substrate feeding in semi-permeabilized cells.

1. Grow hPSCs according to procedures described in Box 3. On the basis of our optimization results, to obtain a robust signal for one run of hPSCs a minimum of 5×10^6 cells is needed, which requires six wells of ~80% confluent hPSCs grown on a six-well Matrigel plate or on a 10-cm plate. To perform the analysis in triplicate, at least three 10-cm plates are required that have been cultured with 10 ml of StemPro medium per plate for 4–5 d with daily medium changes.
2. To collect the cells, aspirate off the StemPro medium. Wash once with $1 \times$ PBS (pH 7.4) and add 1 ml $1 \times$ trypsin-EDTA; incubate for 7 min at 37 °C. Monitor the cells with a phase-contrast light microscope to confirm that the cells are separated into single cells as they detach from the plate surface.
3. Stop the reaction with an equal volume of trypsin inhibitor (1 ml, in this case). Count live cells with trypan blue using a cell counter or other method.
4. Aliquot 5×10^6 cells, spin for 5 min at 200g in a cell culture centrifuge at RT, and resuspend the hPSCs in 300 μ l of respiration buffer prewarmed to 37 °C in a water bath.
5. Before adding cells for analysis, make certain the Clark-type oxygen electrode reading is stable and set to a convenient baseline (the tracing should be flat). Directly transfer the resuspended cells into the calibrated oxygen electrode chamber as quickly as possible. Try to keep the cells at 37 °C during this transfer to avoid potential changes in cell metabolism.
6. Start reading and monitor the graph generated by the Oxygraph software. Wait until the signal value stabilizes for 1–3 min, and then start to record the basal rate of oxygen consumption from the hPSCs that is driven by internal substrate oxidation for 3–5 min.

? TROUBLESHOOTING

7. To deliver specific oxidation substrates into mitochondria inside cells in order to determine the activities of each ETC complex, permeabilize the plasma membrane with 0.003% (wt/vol) digitonin dissolved in respiration buffer. Inject the digitonin stock solution into the oxygen electrode chamber through the hole on the chamber lid. This procedure may require optimization to selectively permeabilize the plasma membrane only, as detailed previously⁴.

8. Measure the ETC complex activities by adding complex-specific substrates while simultaneously inhibiting the activities of preceding complexes in the ETC. In Table 2, we provide different substrate plus inhibitor combinations, which can be used to distinguish the activity of the different respiratory chain complexes or different states of respiration. Different orders of substrate/inhibitor additions can also reveal different mitochondrial functional features. In Table 3, examples of data that can be obtained by altering the order of addition are shown, with numbers indicating the order of each step of addition in a typical analytic run.

? TROUBLESHOOTING

Box 2**Measuring extracellular flux with free fatty acid as the carbon source •
TIMING 3 h**

1. Prepare hPSCs and pre-hydrate the XF24 Extracellular Flux Analyzer cartridge as described in the main PROCEDURE, Steps 1–7. On the day of experiment, warm 50 ml of 1× KHB assay medium at 37 °C and adjust the pH to 7.4 at 37 °C. Gently wash each well of the XF24 V7 cell culture microplate with 1 ml of 1× KHB medium, leaving a residual ~50 µl in the well to prevent cell drying. Then, calculate and add the volume of 1× KHB medium at 37 °C to each well to reach a final volume of 750 µl, which will depend upon the number of fatty acid and inhibitor deliveries that are subsequently injected into each well as described in Step 2 below. Return the plate in a non-CO₂ incubator at 37 °C until loading the plate onto the XF24 Extracellular Flux Analyzer.
2. Prepare the free fatty acid and fatty acid oxidation inhibitor injection solutions. For fatty acid oxidation studies, add 75 µl of 5× sodium palmitate (1 mM) into injection ports A and B (because the limited solubility of palmitate in 1× KHB medium requires a larger volume solution than each injection port can handle). Delivery of both injection port contents into a final volume of 750 µl will make the final palmitate concentration 200 µM. Dilute the 100 mM etomoxir oxidation inhibitor stock solution dissolved in water to 1 mM with 1× KHB buffer to make 10× etomoxir and add 75 µl into injection port C. The final concentration of etomoxir after injection will be 100 µM. To run the XF24 Extracellular Flux Analyzer, follow PROCEDURE Steps 12–16 and use the program shown in Figure 3.
3. Follow PROCEDURE Steps 17–21 to determine protein concentrations and to interpret the data.

Box 3**Maintenance and splitting of hPSCs on Matrigel plates • TIMING 1 h**

hPSCs are initially grown on a MEF feeder layer. To eliminate MEF contamination before experiments, split hPSCs from MEF feeder layer plates to Matrigel-coated plates, as reported in detail previously³⁹ and described below. hPSCs can be maintained on Matrigel-coated plates for approximately ten passages.

1. To coat a six-well plate or a 10-cm culture dish with Matrigel, dilute Matrigel 60-fold with DMEM/F-12 medium on ice. Apply 1 ml of the Matrigel DMEM/F-12 solution to each plate well or 6 ml to a 10-cm culture dish. Let the plate sit under sterile conditions at RT for at least 40 min.
2. Prepare StemPro medium (see REAGENT SETUP) and add bFGF (10 $\mu\text{g ml}^{-1}$ stock diluted to a final concentration of 10 ng ml^{-1}). Prewarm the medium in a 37 °C water bath.
3. Prewarm dispase solution in a 37 °C water bath (see REAGENT SETUP).
4. Before splitting hPSCs, observe colonies under a microscope and mark the colonies that have started to differentiate; these are thus selected for removal.
5. Remove the medium and aspirate off the differentiated colonies with a vacuum-coupled Pasteur pipette and discard. Add 1 ml per well or 3–5 ml per 10-cm culture dish of 1× PBS (pH 7.4) to wash hPSCs.
6. Aspirate off PBS, add 1 ml or 6 ml of warmed dispase solution to each well of a six-well plate or a 10-cm culture dish, respectively. Incubate for 3 min.
 - ▲ **CRITICAL STEP** Do not exceed the incubation time, because the resulting cell clumps could be too small to subsequently attach to the bottom of a well or culture dish; this potentially affects the survival of hPSCs.
7. Take the plate from Step 1 and discard the DMEM/F-12 medium after the Matrigel has coated the plate. Cover the well or plate with desired amount of pre-warmed StemPro medium supplemented with bFGF. The final total volume for one well of a six-well plate is 2 ml and 10 ml for a 10-cm culture dish.
8. Take the plate or dish from Step 6 and remove the dispase. Wash twice with 1× PBS, pH 7.4.
 - ▲ **CRITICAL STEP** Any remaining dispase could potentially inhibit cell attachment and lead to cell death. Also, pipetting after dispase treatment should be done at the edge of the well or dish to prevent loss of loosely attached colonies.
9. Add 1 ml or 3–5 ml of prewarmed StemPro medium containing bFGF to each well of a six-well plate or a 10-cm culture dish, respectively. Use a cell scraper to carefully scrape the colonies from the bottom. Pipette up and down carefully to create medium-sized cell clumps (very often one pipetting step yields the optimal clump size) and split in a desired ratio to prepared StemPro medium-containing Matrigel plates or dishes.
 - ▲ **CRITICAL STEP** The size of the hPSC clump is crucial for optimal growth. Big colonies differentiate easily at late days of passage, whereas small clumps fail to reattach to the Matrigel-coated wells and will eventually die.

Figure 1.
Flowchart of the experimental procedures and estimated time needed for each step.

Protocol commands:

Calibrate

Equilibrate

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

Inject from port A: oligomycin A

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

Inject from port B: FCCP

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

Inject from port C: rotenone + antimycin

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

End

Figure 2.

Flowchart of a typical XF24 analyzer protocol command sequence for measuring extracellular flux with glucose as the carbon source.

Protocol commands:

Calibrate

Equilibrate

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

Inject from port A: palmitate

Inject from port B: palmitate

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

Inject from port C: etomoxir

Mix	2 min	}	Perform 4×
Wait	2 min		
Measure	4 min		

End

Figure 3.

Flowchart of a typical XF24 analyzer protocol command sequence for measuring extracellular flux with free fatty acid as the carbon source.

Figure 4.

OCR, ECAR and OCR/ECAR ratios for hPSCs and human fibroblasts. **(a)** OCR and ECAR values are plotted as a function of the number of cells seeded in each well of a XF24 V7 cell culture microplate. R^2 is the correlation coefficient between OCR or ECAR and the number of cells seeded. A total of 75,000 NHDF cells per well is beyond the linear range of instrument measurements for ECAR. Therefore, an upper cell limit of 50,000 cells for these cell types is recommended for XF24 Extracellular Flux Analyzer studies. **(b)** OCR/ECAR ratio for hESCs (HSF1 and H1), human fibroblasts (NHDF) and hiPSCs (HIPS2 and HIPS18). In total, 20,000 or 35,000 cells were seeded in each well of a XF24 V7 cell culture microplate. Notably, all cell types were prepared identically including attachment to Matrigel-coated plates, culturing in standard StemPro medium supplemented with bFGF and ROCK inhibitor, and incubation on the same XF24 microplate for at least 16 h before medium exchange with the XF24 assay medium. Error bars show mean $\pm$ s.d. ($n = 3$), $*P < 0.05$, with independent cell cultures on different days. Figure is reprinted with permission from ref. 14.

Figure 5. Bioenergetic profile of hPSCs and human fibroblasts. **(a)** Percentage OCR changes for hPSCs (HSF1) and NHDF cells plotted against time during a XF24 Extracellular Flux Analyzer run. Oligomycin A (1 μ M), 0.3 μ M FCCP and 1 μ M rotenone + 1 μ M antimycin A (final concentrations) were sequentially delivered to the XF24 assay medium through injection ports in the sensor cartridge. **(a,b)** The dashed lines in **a** represent the average value of multiple time point measurements and the numbers indicate the bioenergetic parameters discussed in **b**. **(b)** Summary of the data from **a**. OCR consumed for making ATP is the difference between the starting basal OCR at 2 min and oligomycin A–repressed OCR. The residual OCR, determined as the difference in OCR between oligomycin A and rotenone + antimycin A exposures, is due to a nonspecific proton leak across the mitochondrial inner membrane. The maximal/initial OCR is the ratio between FCCP uncoupled OCR and basal OCR at 2 min. Error bars show mean $\pm$ s.d. ($n = 3$), $*P < 0.05$, with independent cell cultures on different days. Part of this figure is reprinted with permission from ref. 14.

Figure 6.

Comparison of data obtained with the XF24 Extracellular Flux Analyzer and conventional energy metabolism profiling methods. **(a)** Comparison of OCR values obtained for hPSCs (HSF1) in the basal state using a Clark-type oxygen electrode and the XF24 Extracellular Flux Analyzer. OCR for both methods obtained from three independent cell culture preparations. Values are mean $\pm$ s.d. ($n = 3$). The single-cell values in the last column are calculated from data in the preceding columns. **(b)** A polarograph tracing demonstrates specific ETC complex activities with 5×10^6 permeabilized HSF1 cells. The fluctuation at ~ 2 min is caused by exchanging the instrument buffer with a cell suspension. Digitonin (0.003% (wt/vol)) was added to permeabilize the cells at the indicated time point. ETC complex activities were measured by adding complex-specific substrates after inhibiting prior complexes in the chain. The initial activity of the respiratory complexes, starting with complex I, is measured by adding 8 mM pyruvate (or 15 mM glutamate) + 2.5 mM malate. Complex II–IV activities are measured by adding 10 mM succinate after inhibiting complex I with 3.3 μ M rotenone. Complex III–IV activities are measured by adding 20 mM glycerophosphate after inhibiting complex II with 10 mM malonate. Complex IV activity is measured by adding 10 mM ascorbate + 0.2 mM TMPD (tetramethyl-*p*-phenylenediamine) after inhibiting complex III with 33 mM antimycin A. KCN is an inhibitor of complex IV that stops oxygen consumption. CCCP is used to stimulate maximal respiratory capacity by uncoupling proton pumping. **(c)** ECAR measured with the XF24 Extracellular Flux Analyzer compared with lactate content in the XF24 assay medium determined with a lactate assay kit. The samples for lactate determination (right) were taken from the same wells in the XF24 V7 cell culture microplate as used for ECAR determinations (left). HSF1 cells were differentiated with 10 μ M retinoic acid for 9 d. Error bars show mean $\pm$ s.d. ($n = 3$), $*P < 0.05$. Part of the figure is reprinted with permission from ref. 14.

Figure 7.

Free fatty acid oxidation in hPSCs and human fibroblasts determined with the XF24 Extracellular Flux Analyzer. **(a)** OCR of 35,000 hPSCs (H1) and human fibroblasts (NHDF) was measured. Cells were incubated sequentially with 200 μ M sodium palmitate, followed by 100 μ M etomoxir. The dashed lines represent the average value of multiple measurements before sodium palmitate addition. The dotted lines represent the measured values after sodium palmitate or etomoxir additions, respectively. **(b)** Percentage change in OCR of HSF1 and NHDF cells with increasing concentrations of added sodium palmitate. Three replicate experiments prepared from a single cell culture were measured independently. Error bars show mean $\pm$ s.d. ($n = 3$), $*P < 0.05$. Part of the figure is reprinted with permission from ref. 14.

Figure 8.

Using the XF24 Extracellular Flux Analyzer with a wide variety of mammalian cell types. **(a)** Dependence on respiration or glycolysis of various cell types shown by plotted OCR/ECAR ratios. Poly-L-lysine (0.01% (wt/vol)) was coated on XF24 assay plate wells for 1 h to facilitate nonadherent blood cell attachment. **(b)** Absolute OCR and ECAR values for 50,000 cells of various types. Dashed line is an arbitrary line that separates mainly glycolytic from mainly respiration-dependent cell types. Three replicate experiments prepared from a single-cell culture were measured independently. Error bars show mean $\pm$ s.e.m. ($n = 3$).

Table 1

Common bioenergetic analysis methods.

Method	Principle	Advantages/disadvantages
<i>Extracellular flux (O_2 consumption, lactate excretion) analysis methods</i>		
Traditional Clark-type oxygen electrode (e.g., Hansatech Instruments)	Oxygen dissolved in liquid or gas phase in the sample chamber is detected by polarography using a Clark-type oxygen electrode ^{47,48}	Advantages: flexibility in measuring mitochondrial functions (see Table 2). Isolated mitochondria, permeabilized cells or intact cells can be used, and large numbers of substrates, inhibitors or uncouplers can be easily added Disadvantages: oxygen continuously leaks from the apparatus, time-consuming system assembly, some drugs are not easily removed and intensive chamber washing may be required, frequent calibration required, oxygen-sensing electrode typically has a short lifetime, and large sample sizes required
Oroboros Oxygraph-2k (Oroboros Instruments)	Oxygen dissolved in liquid or gas phase in the sample chamber is detected by polarography using a Clark-type oxygen electrode with high-resolution respirometry ^{4,49}	This is an advanced polarimeter that has two chambers for parallel measurements Advantages: instrument setup and calibration need to be done every few months, oxygen leak from instrument is reduced, increased detection sensitivity and small samples can be used Disadvantages: instrument cost
XF24 Extracellular Flux Analyzer (Seahorse Bioscience)	Detection of OCR with oxygen-sensing fluorophores and ECAR with pH sensor simultaneously in the same population of intact cells ⁵⁰	Advantages: small sample sizes (e.g., $\sim 10^4$ intact cells), OCR and ECAR in parallel 24-well or 96-well plate measurements, allowing for replicates and multiple conditions, sensors in close proximity to cells increasing accuracy, plates/cartridges are disposable Disadvantages: instrument and consumable plate costs, limited number and type of delivered modifiers
Fiber optic oxygen sensors (e.g., Ocean Optics Sensors)	A fluorescence method is used to measure the partial pressure of dissolved or gaseous oxygen in a sample ⁵¹	Advantages: long sensor lifetime (>1 year) and does not consume oxygen, allowing for extended measurements of samples
<i>Additional common complementary bioenergetic analysis methods</i>		
ATP assays (e.g., CellTiter-Glo Luminescent Cell Viability Assay, Promega) ADP/ATP ratio (e.g., ApoSENSOR ADP/ATP Ratio Assay Kit, Biovision)	Bioluminescent detection of luciferase activity that is proportional to the ATP concentration in a sample ⁵²	Advantages: detection of whole-cell steady-state ATP levels or ADP/ATP ratios Disadvantages: mechanisms contributing to ATP level cannot be dissected and, because of typically rapid changes in ATP/ADP levels, sensitivity is limited to large changes in concentrations
Mitochondrial membrane potential ($\Delta\psi$)-detecting dyes (e.g., tetramethyl rhodamine ethyl ester, Invitrogen)	Fluorescence detection with dyes that are usually cell permeant, cationic and readily sequestered by active mitochondria with a proton gradient at the mitochondrial inner membrane ⁵³	Advantages: a quick, easy method for measuring Disadvantages: dyes can inhibit the ETC complexes, requiring control titrations for optimal concentrations
Stable isotope tracing analysis/metabolomics	Stable isotope-labeled carbon substrates, such as ^{13}C -labeled glucose, are fed to cells, followed by tracing of intermediate metabolite isotopomers using mass spectrometry ⁵⁴	Advantages: approach systematically analyzes the metabolic fate of specific carbon sources Disadvantages: instrument expense, frequently requires expertise in metabolomics and large data set analysis

Table 2

Agents used to dissect ETC activities in isolated mitochondria with a Clark-type oxygen electrode.

Agent	Description	Time
8 mM pyruvate + 2.5 mM malate	Measures OCR from combined ETC complexes I, III and IV. Electrons from NADH generated from pyruvate oxidation in the TCA cycle enter the respiratory chain via complex I. Malate enters mitochondria via the malate-aspartate shuttle and supports the TCA cycle. This measures basal cell respiration rate	~5 min
15 mM glutamate + 2.5 mM malate	Measures OCR from combined ETC complexes I, III and IV. Electrons from NADH generated from glutamate oxidation by glutamate dehydrogenase enter the respiratory chain via complex I	~5 min
50 μ M ADP	When the ETC is coupled to ATP synthesis, ADP addition will increase the OCR in proportion to the ADP addition. Protons from the ETC that have accumulated in the mitochondrial intermembrane space will pass through complex V when ADP is added. It is crucial that P_i is present in the oxygen electrode sample buffer to synthesize ATP from the added ADP	~2–5 min
3.3 μ M rotenone	Rotenone inhibits ETC complex I, so the OCR will be reduced to the level representing other O_2 -consuming processes in the system	~2–5 min
10 mM succinate	Succinate provides electrons for ETC complex II and bypasses ETC complex I. OCR increases through complex II when mitochondria are also exposed to rotenone	~2–5 min
10 mM malonate	Malonate competitively inhibits ETC complex II. The extent of OCR inhibition by malonate indicates the activity and intactness of ETC complex II. It is crucial that Complex II inhibition by malonate is relatively short lived because it is a reversible inhibitor	~1–2 min
20 mM glycerol-3-phosphate (G3P)	G3P provides electrons for ETC complex III through glycerol-phosphate cycle by glycerol-3-phosphate dehydrogenase and bypasses blocked complexes I and II; OCR increases through complex III when mitochondria are also exposed to malonate plus rotenone	~2–5 min
33 nM antimycin A	Antimycin A inhibits ETC complex III. Addition of antimycin A after G3P is useful for addressing the OCR activity and intactness of ETC complex III	~2–5 min
10 mM ascorbate + 0.2 mM TMPD (tetramethyl- <i>p</i> -phenylenediamine)	TMPD is an artificial electron donor to ETC complex IV and ascorbate maintains TMPD in a reduced state. OCR increases through complex IV when mitochondria are also exposed to antimycin A	~2–5 min
3.3 mM KCN	KCN inhibits ETC complex IV and is also toxic for cells. OCR should decrease almost to the calibrated instrument baseline	~1–2 min
0.3 μ M FCCP 0.3 μ M CCCP	FCCP and CCCP are ETC uncouplers that dissipate the mitochondrial inner membrane potential by acting as a proton ionophore to generate a nonspecific proton leak. They are used to determine maximal respiratory capacity by uncoupling oxygen consumption from ATP production in the ETC complex V F_1F_0 ATP synthase	~1–2 min

Table 3

Order of agent addition for analyzing ETC functions in isolated mitochondria with a Clark-type oxygen electrode.

Agent added	ETC coupling with ATP synthesis	State $\frac{3}{4}$ (also reflects coupling)	Stepwise control of the ETC-CI linked	Stepwise control of the ETC-CII linked	CIII-CI linked	CIV-CI linked
Malate	(1)	(1)	(1)		(1)	(1)
Glutamate	(1)	(1)	(1)		(1)	(1)
ADP		(2)				
FCCP	(3)	(3)				
Rotenone			(2)			
Succinate			(3)	(1)		
Malonate			(4)	(2)		
G3P			(5)	(3)		
Antimycin A			(6)	(4)	(2)	
TMPD			(7)	(5)		(2)
Ascorbate			(7)	(5)		(2)
Oligomycin	(2)					
KCN	(4)	(4)	(8)	(6)	(3)	(3)

To address different questions, the table lists several options for Clark-type oxygen electrode studies. The first option, 'ETC coupling with ATP synthesis' investigates whether respiration is coupled to ATP synthesis, the maximum respiration capacity and additional oxygen-consuming pathways. This setup is useful for the XF24 analyzer as well. Figure 5a,b presents typical results for this setup using the XF24 analyzer. The second option includes the addition of ADP to measure ADP-induced respiration in isolated mitochondria. Basal respiration versus ADP-induced respiration provides a 'coupling factor' for the respiratory chain and ATP synthesis. This factor is an indication of the intactness of mitochondria. The third and following options, which use the addition of individual substrates and inhibitors that target specific ETC complexes, allow assessments of the activities of specific ETC components. Low oxygen consumption could be from a defect in a particular component of the ETC pathway, for example.

Table 4

Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Poor survival of seeded hPSCs	Poor maintenance of cell culture on Matrigel plates before seeding	Thaw a new vial of hPSCs, initially plate and grow on a MEF monolayer. After colonies grow to 70–80% confluence, split cells onto Matrigel plates and grow for at least one more passage. Do not use hPSCs on Matrigel plates for more than 10 passages
		Poor quality of ROCK inhibitor	Use a fresh aliquot of ROCK inhibitor stored at – 20 °C for no longer than 1 month. Do not reuse thawed aliquots
	hPSCs do not form a uniform monolayer at the bottom of the XF24 microplate wells	hPSC clumps did not dissociate into single cells	Use fresh trypsin, avoid leaving trypsin at RT for a long time, increase the time of trypsin treatment to 10 min
		Pipette tip is not against the lower wall of the well when injecting cells, or cells did not have enough time to attach between the first and second injections of XF24 assay medium	Inject 100 µl of cell suspension against the lower wall first, let cells settle for at least 1 h, and then inject 100 µl of XF24 assay medium against the upper wall of the well. You can use a 96-well plate to practice seeding and observe the cells under a microscope the next day
15	Low signal detection for OCR or ECAR measurements	Low rate of hPSC survival	Use fresh ROCK inhibitor and reduce the time for cell splitting.
		Not enough time for XF24 analyzer measurements	In the protocol commands, increase ‘measurement’ from 4 to 5 min
		Poor cartridge probes	Check both oxygen and pH sensors before experiments, immerse both sensors into XF calibrant solution in each well overnight, and wrap the cartridge with Parafilm to prevent solution evaporation and drying
		Oxygen leak	Use the corrected algorithm ⁵⁵ . In the XF24 software, click ‘Special operations’, then click ‘Change O ₂ calculation method, then choose ‘AKOS’
	Inaccurate measurements of OCR or ECAR	Number of hPSCs seeded is greater than the linear range	Titrate hPSCs from 20,000 to 75,000 cells per well, plot OCR and ECAR against number of cells seeded. See ANTICIPATED RESULTS and Figure 4a
		hPSCs do not form uniform monolayers in assay wells	Titrate cells from 20,000 to 75,000 cells per well with good seeding techniques, as described above
		Background reading is high	Leave one well unseeded, apply all the procedures to this well, and set it as ‘blank’ when running the XF24 analyzer data collection program
		pH is not controlled	Use unbuffered DMEM to make the assay medium. Adjust pH after adding drugs at 37 °C. Do not put either cartridge or microplate into a CO ₂ incubator
19	Inaccurate protein concentrations	Loss of cells due to low attachment	Some cells attach loosely to the bottom of the assay wells, especially after a series of inhibitor or uncoupler exposures. Gently wash wells before adding lysis buffer. For cell types that do not need Matrigel and attach loosely, such as HEK293T cells, use 100 µl of 0.01% (wt/vol) poly- <i>L</i> -lysine to precoat assay wells for 1 h before seeding cells to increase attachment
20	Low maximal respiratory capacity	Low concentration of carbon substrate(s) in the assay medium	Titrate concentrations of glucose up to 25 mM and pyruvate up to 2 mM in the assay medium. Add insulin to see whether maximal respiratory capacity can be further induced, because insulin may stimulate glucose uptake in certain cell types
Box 1 (Step 6)	OCR signal detection is barely above background	Background signal is not set to 0	Use fresh filtered and prewashed respiration buffer and wait until the signal becomes flat. For a newly assembled Clark-type oxygen electrode, the stabilization period could be as long as several hours. Carefully clean the oxidized silver precipitate on the electrode after each run to reduce the stabilization time for future instrument runs
		Not enough cells (< 5 × 10 ⁶	Increase cell numbers

Step	Problem	Possible reason	Solution
Box 1 (Step 8)	Inhibitors or substrates added into the electrode sample chamber do not work	cells per run can greatly affect the signal-to-noise ratio)	
		Cells are not permeabilized	Optimize choices/concentration of detergents, such as digitonin or saponin
		Old stocks; pH is not adjusted	Prepare new stocks and adjust each pH to 7 with pH paper
		Cross-contamination of Hamilton syringes used for injections	Dedicated, individual Hamilton syringes are suggested for each ETC substrate, inhibitor and uncoupler to eliminate cross-contamination