

Rapid immunopurification of mitochondria for metabolite profiling and absolute quantification of matrix metabolites

Walter W Chen^{1–5}, Elizaveta Freinkman^{1,6} & David M Sabatini^{1–4}

¹Department of Biology, Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. ²Department of Biology, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. ³Koch Institute for Integrative Cancer Research, Cambridge, Massachusetts, USA. ⁴Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. ⁵Harvard Medical School, Boston, Massachusetts, USA. ⁶Present address: Metabolon, Inc., Research Triangle Park, North Carolina, USA. Correspondence should be addressed to D.M.S. (sabatini@wi.mit.edu).

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Mitochondria carry out numerous metabolic reactions that are critical to cellular homeostasis. Here we present a protocol for interrogating mitochondrial metabolites and measuring their matrix concentrations. Our workflow uses high-affinity magnetic immunocapture to rapidly purify HA-tagged mitochondria from homogenized mammalian cells in ~12 min. These mitochondria are extracted with methanol and water. Liquid chromatography and mass spectrometry (LC/MS) is used to determine the identities and mole quantities of mitochondrial metabolites using authentic metabolite standards and isotopically labeled internal standards, whereas the corresponding mitochondrial matrix volume is determined via immunoblotting, confocal microscopy of intact cells, and volumetric analysis. Once all values have been obtained, the matrix volume is combined with the aforementioned mole quantities to calculate the matrix concentrations of mitochondrial metabolites. With shortened isolation times and improved mitochondrial purity when compared with alternative methods, this LC/MS-compatible workflow allows for robust profiling of mitochondrial metabolites and serves as a strategy generalizable to the study of other mammalian organelles. Once all the necessary reagents have been prepared, quantifying the matrix concentrations of mitochondrial metabolites can be accomplished within a week.

INTRODUCTION

Mitochondria are membrane-bound organelles that house numerous chemical reactions that are essential to cellular metabolism and mammalian physiology^{1–9}. Importantly, the metabolic milieu of the mitochondrial matrix compartment is distinct from that of the rest of the cell because of the mitochondrial inner membrane. Yet efforts to systematically profile the small-molecule contents of mitochondria in mammalian cells have been limited. In recent years, mass spectrometry has greatly facilitated the study of cellular metabolism by enabling large-scale assessments of the metabolite composition of whole cells¹⁰. However, metabolite profiling of whole cells fails to capture the dynamics of metabolic reactions and metabolite changes within the mitochondrial matrix, as this compartment represents only a small portion of the total cellular material.

Here we present a detailed workflow for the rapid isolation of mitochondria from mammalian cells and the quantification of matrix concentrations of mitochondrial metabolites (Fig. 1). The method uses high-affinity magnetic immunopurification (IP) of HA-tagged mitochondria to achieve rapid isolation of mitochondria within 12 min following cellular homogenization. Mitochondrial metabolites are interrogated using LC/MS, and matrix concentrations are calculated through a combination of LC/MS, immunoblotting, confocal microscopy, and volumetric analysis.

Development of the protocol

Metabolite profiling of intracellular organelles such as mitochondria is technically challenging because the isolation method must be both fast and specific. At low temperatures, inner-membrane

solute transporters and matrix enzymes can still exhibit residual activity, leading to distortion of the metabolite profile during long isolation procedures^{11–13}. Standard methods for purifying mitochondria can take hours to complete and are thus too slow for profiling the metabolic contents of mitochondria. Existing methods that offer accelerated isolation procedures can suffer from poor purity, resulting in metabolic extracts that contain not only mitochondrial metabolites but also cytosolic, lysosomal, and peroxisomal contents^{12,14–21}. As such, interrogation of the metabolic contents of mitochondria requires a method that is both rapid and specific.

An additional challenge is that existing mitochondrial isolation buffers contain large amounts of solutes (e.g., sucrose, mannitol) that are not compatible with LC/MS¹⁹. Because of the sensitivity of LC/MS instruments to small-molecule contaminants, these solutes can interfere with the analysis of mitochondrial metabolites. In certain cases, such contamination can completely prevent detection of a metabolite²¹.

To address these challenges, we developed a workflow using high-affinity magnetic immunocapture that is both LC/MS-compatible and capable of rapidly and specifically isolating mitochondria for metabolite profiling²¹. Because small molecules interfere with LC/MS analysis, we designed a mitochondrial isolation buffer composed of only KCl and KH₂PO₄, which we named 'KPBS'. We found that KPBS maintained mitochondrial integrity, which is consistent with previous literature using KCl-based buffers²², and also markedly improved the quality of our LC/MS data²¹.

To achieve rapid and specific isolation of mitochondria, we used a strategy whereby an outer mitochondrial membrane

PROTOCOL

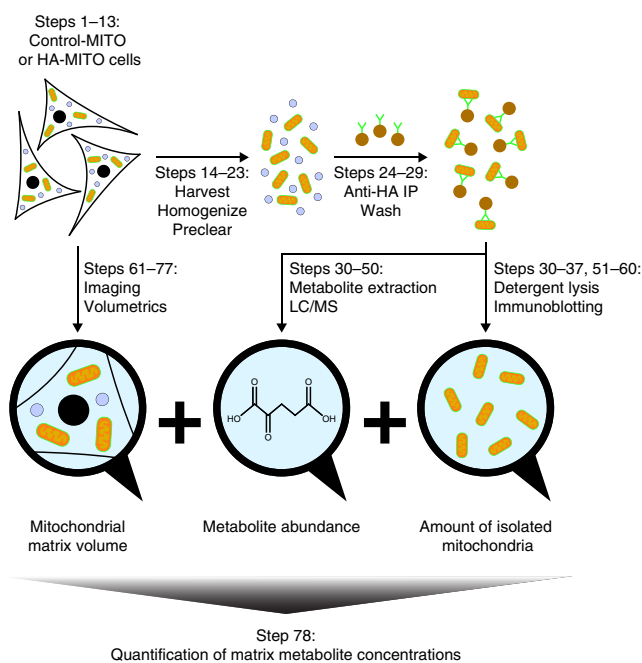


Figure 1 | Workflow for quantifying matrix concentrations of mitochondrial metabolites. Step numbers in the figure correspond to those in the PROCEDURE. Cells expressing appropriate amounts of the *3XMyc-EGFP-OMP25* gene (Control-MITO cells) or the *3XHA-EGFP-OMP25* gene (HA-MITO cells) are generated using retroviral transduction and fluorescence-activated cell sorting in Steps 1–13. Control-MITO or HA-MITO cells are quickly harvested and homogenized, with the homogenate precleared to remove cells, nuclei, and other large debris, resulting in a suspension of mitochondria and other organelles (Steps 14–23). After a short anti-HA immunoprecipitation (IP) (3.5 min in length) to capture HA-tagged mitochondria, antibody-conjugated beads are quickly washed three times (Steps 24–29). The majority of the isolated mitochondria are extracted for metabolites and the corresponding mole quantities are determined by liquid chromatography and mass spectrometry (LC/MS) (Steps 30–50). The remaining isolated mitochondria are then lysed for protein, and whole-cell equivalents of mitochondria in each IP sample are determined by immunoblot analysis (Steps 30–37, 51–60). Confocal microscopy and volumetric analysis of HA-MITO cells is used to quantify the total mitochondrial volume per cell, which is then adjusted using the percentage of mitochondrial volume occupied by the matrix (~63.16% of mitochondrial volume = matrix)⁴² (Steps 61–77). The matrix concentration of a mitochondrial metabolite is derived from the combination of all of these measurements (Step 78).

protein could serve as a handle for immunocapture. Taking advantage of the high affinity and specificity of the interactions between the HA epitope and its cognate antibody, we designed a chimeric protein comprised of three HA epitope tags joined to the N terminus of EGFP, which is then fused to the outer mitochondrial membrane localization sequence of OMP25 (ref. 23). After extensive optimization of the IP system, we found that traditional beads used for protein immunocapture are too large (~50- μm diameter) and not well suited to mitochondrial isolation. Although their porous matrix is designed to enhance immunocapture by generating substantial amounts of internal surface area, these beads have pores (~30-nm diameter) that are probably too small for mitochondria (~500-nm diameter) to enter. As such, the majority of the immunocapture is probably limited to the surface of these beads, in which case reducing the size of each individual bead should increase the yield considerably. Indeed,

reducing the size of beads from a diameter of 50 to 1 μm markedly increases the amounts of mitochondria that are isolated²¹. Collectively, these improvements allow our LC/MS-compatible methodology to rapidly purify mitochondria within 12 min of homogenizing the cells, thereby allowing for robust interrogation of mitochondrial metabolites.

To quantify matrix concentrations of mitochondrial metabolites, we developed a workflow that integrates LC/MS-based mitochondrial profiling with immunoblotting, confocal microscopy, and volumetric analysis (Fig. 1). The mole quantity of a mitochondrial metabolite in the IP material is determined by LC/MS using authentic metabolite standards and isotopically labeled internal standards, whereas the total matrix volume per cell is assessed by confocal microscopy of intact cells and volumetric analysis. Using citrate synthase (CS), a protein localized specifically to the mitochondrial matrix²⁴, immunoblot analyses of whole-cell and IP lysates allow one to calculate the number of whole-cell equivalents of isolated mitochondria. In conjunction with imaging and volumetric analysis of mitochondria within intact cells, this information is then used to determine the corresponding matrix volume of the isolated mitochondria. The matrix concentration of a metabolite is then calculated using the mole quantity of that metabolite and the corresponding matrix volume.

Importantly, we show in our previous study that mitochondria isolated via this workflow possess notable integrity with respect to matrix metabolites²¹. By taking advantage of the fact that both the protein CS and the metabolite coenzyme A (CoA) are predominantly localized to the mitochondrial matrix in cells^{25,26}, we find that estimates of mitochondrial abundance in the IP material using either CS or CoA lead to similar results. These data demonstrate that we are obtaining mitochondria with inner membranes that are intact with respect to both proteins and metabolites, an important feature of any method designed to profile the mitochondrial metabolome²¹.

Applications of the method

The workflow described here allows for profiling of mitochondrial metabolites and quantification of their matrix concentrations. As described previously, this methodology can have substantial utility in understanding the metabolic changes occurring within the matrix compartment of cultured cells under different experimental conditions^{21,27,28}. From our work studying the effects of respiratory chain (RC) inhibition, we have found that metabolites can behave in substantially different ways when assessed using mitochondrial metabolomics versus traditional whole-cell metabolomics. For example, following the acute inhibition of RC complexes I, III, and V, whole-cell levels of phosphoenolpyruvate (PEP) appear relatively unchanged, but matrix levels of PEP are markedly lowered. This difference is attributable to the fact that the PEP pool within mitochondria can be a small portion of the whole-cell PEP signal and can also be generated by different metabolic pathways than those used for making extra-mitochondrial PEP^{21,29}. Collectively, these prior experiments demonstrate the importance of mitochondrial profiling, and thus the workflow described here should facilitate the investigation of how mitochondria respond to different types of perturbations.

In addition, the ease of introducing the necessary mitochondrial tagging constructs into cells should make extending this

technique to different cell lines relatively straightforward. In cell types in which viral transduction is undesirable, these constructs can also be transfected into cells before mitochondrial isolation. Moreover, the use of epitope tags enables the isolation of mitochondria from specific cell types, a particularly valuable feature in mixed-cell culture systems and *in vivo*.

Because the epitope-tagging strategy used in this workflow is quite generalizable, additional applications of this method include rapid purification of other organelles. Indeed, as long as there exists a protein or amino acid sequence that gives specific localization of the epitope tag to the desired location, this method can be used to profile the metabolic contents of various intracellular organelles that also have important roles in mammalian physiology, such as the lysosome and the peroxisome. Future efforts in which different organelles are profiled in the same cell type will allow for a clearer picture of how metabolism across subcellular compartments is coordinated to achieve cellular homeostasis.

Comparison with other methods

As mentioned previously, the workflow described here allows for isolation of mitochondria with both rapidity and high specificity, two requirements for faithful interrogation of mitochondrial metabolites. Prior efforts studying the metabolic contents of mitochondria have relied on methods that generally prioritize one requirement at the expense of the other. For example, isolation methods that can yield relatively pure mitochondria, such as sucrose-gradient centrifugation^{30,31} or the Miltenyi immunopurification kits, take notably longer to complete than our workflow, leading to greater distortion of the mitochondrial metabolite profile. Abbreviated forms of centrifugation, nonaqueous fractionation, and selective membrane permeabilization all isolate mitochondria with great speed, but the resulting material can be contaminated with whole cells and other organelles such as lysosomes and peroxisomes^{12,14–20}. Lysosomes in particular can distort the metabolite profile of mitochondrial preparations because they contain a notable amount of amino acids (data not shown). Taken together, the improvements in both speed and specificity of the workflow described here make it well suited for profiling mitochondrial metabolites.

Experimental design

An important principle of this workflow is that each experimental condition requires an anti-HA IP using both cells expressing the *3XMyC-EGFP-OMP25* (Control-MITO) gene and cells expressing the *3XHA-EGFP-OMP25* (HA-MITO) gene. These genes can be introduced into any cell type amenable to transfection or viral transduction. The Control-MITO IP is important, as it can help distinguish true mitochondrial metabolites from metabolites that are retained nonspecifically on the beads. As such, Control-MITO and HA-MITO IP samples are all analyzed in the same batch on the LC/MS instrument so that any metabolite signals present in the Control-MITO IP sample can be subtracted from the corresponding signals in the HA-MITO IP sample during data processing. In general, metabolites that are not at least 1.5-fold more abundant in the HA-MITO IP sample than in the Control-MITO IP sample are considered background and not mitochondrial.

In addition to correcting for the background metabolite signal, determining how much extra-mitochondrial material is present in the HA-MITO IP sample is also important for assessing the

quality of the data. Immunoblot analysis of isolated mitochondria with markers of different subcellular compartments is very useful for interrogating the contributions of organelles such as lysosomes and peroxisomes. Complete elimination of all extra-mitochondrial contamination is very difficult in any organellar purification method, but one should see the ratio of mitochondrial markers to extra-mitochondrial markers increase in the IP as compared with the whole-cell material. Additional controls for assessing the purity of the IP also include measurement of the amount of metabolites thought to be predominantly in extra-mitochondrial compartments, such as fructose 1,6-bisphosphate (cytosol) and cystine (lysosomes).

In terms of technical difficulty, although the workflow described here involves multiple stages of analysis, it is not overly difficult to implement. Indeed, the methodology uses materials, equipment, and facilities accessible to many academic laboratories. However, it should be mentioned that large experiments using this method can require a notable amount of time to complete. Plates of cells are processed one at a time to facilitate the speed of mitochondrial isolation, and each experimental condition requires at least three biological replicate sets of both a Control-MITO IP and an HA-MITO IP. Furthermore, absolute quantification of more than 100 metabolites requires a considerable amount of LC/MS instrument time and data analysis because of the need for standard curves. However, for certain experiments, relative, rather than absolute, quantification of metabolites is probably sufficient, in which case LC/MS standard curves do not need to be included in every run, and confocal microscopy and volumetric analysis of each experimental condition is not required, thus shortening the workflow considerably.

Level of expertise needed to implement the protocol. A graduate student or postdoctoral researcher can perform all steps of this protocol, but a fluorescence-activated cell sorting (FACS) core facility usually operates the FACS machine and a metabolite profiling core facility typically operates the LC/MS instrument and performs the LC/MS analysis. If a metabolomics facility is unavailable on-site, then samples can also be shipped on dry ice to institutions with metabolite profiling capabilities. It is also important to note that it can take several rounds of performing the mitochondrial isolation to become adept enough to perform the workflow at its intended speed.

Limitations. Although this workflow is generally robust and quite reproducible, there are some technical points to consider. Thermo Fisher Scientific offers both 1- μ m anti-Myc and 1- μ m anti-HA beads that initially seem equivalent for organellar purification. However, we have found that the anti-Myc beads can actually bind to mitochondria liberated from cells that do not even express the *3XMyC-EGFP-OMP25* construct. Anti-HA beads do not suffer from this limitation, and so we recommend using the anti-HA beads, but not the anti-Myc beads, for organellar capture. Another technical point to consider is that although the HA-MITO IP sample has substantially reduced amounts of extra-mitochondrial material compared with whole cells, we have found that our workflow removes endoplasmic reticulum (ER) less efficiently than lysosomes and peroxisomes. This is probably because the mitochondria and ER form physical contacts mediated by protein–protein interactions^{32–34}. It should also be noted that the

degree of ER carryover probably varies across cell lines depending on the strength of mitochondrial–ER contacts. Thus, although the mitochondria/ER ratio is indeed higher in the HA-MITO IP material than in whole cells, there can still be some ER material that is carried through the workflow.

Because we developed this method using immortalized, proliferating cultured cells, potential limitations can also arise when trying to directly apply this workflow to the study of cells that proliferate slowly, do not proliferate at all, or do not survive for long periods of time in culture. The ideal cellular input for a Control-MITO IP or HA-MITO IP is ~30 million cells. However, acquiring this number of cells can be difficult for certain cell lines or primary cell types. Although we have been able to study successfully matrix metabolites using < 30 million cells, it is worth emphasizing that less abundant metabolites may become undetectable, particularly if they are intrinsically more difficult to detect using LC/MS (i.e., less ionizable). In instances in which detection is poor because of decreased starting material, we have

had success with targeted selected ion monitoring (tSIM) scans, which can improve metabolite signals considerably on the LC/MS system; metabolites that have notably benefited from tSIM scans include certain nucleotide species such as CMP and GMP.

In our prior work, we showed that lengthening the posthomogenization time preceding metabolite extraction from 12 to 16 min does not lead to a substantial distortion of the matrix metabolite profile²¹, but it is worth noting that there are certain mitochondrial metabolites that are more labile than others. For example, the three mitochondrial metabolites that have the greatest fold decrease upon lengthening the workflow are citrate/isocitrate, dihydroxyacetone phosphate (DHAP), and ATP, whereas the three mitochondrial metabolites that have the greatest fold increase upon lengthening the workflow are methionine sulfoxide, asparagine, and cytidine (**Supplementary Data 1**). Although it is difficult to predict the behavior of the more labile metabolites at time points earlier than 12 min, our work suggests that studies of these metabolites could benefit from future improvements in the speed of isolation.

MATERIALS

REAGENTS

- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M6250)
- Acetonitrile, hypergrade for LC/MS (EMD Millipore, cat. no. 100029)
 - ! **CAUTION** Acetonitrile is a highly flammable liquid and vapor.
- Acrodisc syringe filters, 0.45 μm (Pall Life Sciences, prod. no. 4184)
- Ammonium carbonate (Sigma-Aldrich, cat. no. 379999)
- Ammonium hydroxide solution, 28% (wt/vol) ammonia in water (Sigma-Aldrich, cat. no. 338818)
 - ! **CAUTION** Ammonium hydroxide is corrosive and toxic.
- Antibodies against CS (cat. no. 14309), RPS6KB1 (cat. no. 2708), CALR (cat. no. 12238), GOLGA1 (cat. no. 13192), and CAT (cat. no. 12980) are from Cell Signaling Technology. Antibodies against LAMP2 (cat. no. sc-18822), CTSC (cat. no. sc-74590), and LMNA (cat. no. sc-20680) are from Santa Cruz Biotechnology.
- Anti-HA magnetic beads (Thermo Fisher Scientific, cat. no. 88837)
 - ▲ **CRITICAL** We have had the most success using this brand of anti-HA beads, owing to their small size, which we believe greatly enhances the degree of immunocapture for organelles, as described earlier. Anti-Myc magnetic beads (Thermo Fisher Scientific, cat. no. 88843) do not perform as well, as they can nonspecifically capture mitochondria in the absence of a Myc-epitope.
- Blasticidin solution (InvivoGen, cat. no. ant-bl-1)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- CAPS (Sigma-Aldrich, cat. no. C2632)
- Cell scrapers (Corning, cat. no. 3008)
- Circular glass coverslips (Electron Microscopy Sciences, cat. no. 72231-01)
- CL-XPosure films (Thermo Fisher Scientific, cat. no. 34091)
- Cryogenic vials, 2 ml (Corning, cat. no. 430488)
- DMSO (EMD Millipore, cat. no. MX1458-6)
- DMEM powder (Sigma-Aldrich, cat. no. D7777)
- Ethanol, 190 proof (Pharmco-Aaper, cat. no. AAP-111000190CSGL)
 - ! **CAUTION** It is a highly flammable liquid and vapor.
- EDTA solution, 500 mM, pH 8.0 (Thermo Fisher Scientific, cat. no. AM9260G)
 - ! **CAUTION** EDTA is toxic, so avoid exposure.
- BenchMark FBS, heat inactivated (IFS; Gemini Bio-Products, cat. no. 100-106)
- Filter-tops, 0.22-μm filter, 45-mm diameter (Corning, prod. no. 430626)
- Filter-tops, 0.45-μm filter, 33-mm diameter (Corning, prod. no. 430625)
- Filter-top bottles, 0.22-μm filter (Corning, prod. no. 430769)
- Gel-loading tips (Thermo Fisher Scientific, cat. no. 05-408-151)
- Glycerol (EMD Millipore, cat. no. GX0185-6)
- Glycine (Sigma-Aldrich, cat. no. G7126)
- HeLa cells (ATCC, cat. no. CCL-2). We describe how to carry out this protocol using HeLa cells but, as discussed in the INTRODUCTION, other cells can be used
 - ! **CAUTION** Ensure that the cells used in your research remain free of mycoplasma, and regularly check the authenticity of your cells in

- culture ▲ **CRITICAL** The workflow may require minor modifications if using different cell types.
- HEK293T cells (GenHunter, cat. no. Q401)
 - ! **CAUTION** Ensure that the cells used in your research remain free of mycoplasma, and regularly check the authenticity of your cells in culture ▲ **CRITICAL** HEK293T cells are used for virus production.
- HRP-conjugated goat anti-mouse (cat. no. sc-2055) and anti-rabbit (cat. no. sc-2054) secondary antibodies are from Santa Cruz Biotechnology.
- Hydrochloric acid (HCl; EMD Millipore, cat. no. HX0603-4)
 - ! **CAUTION** HCl is highly corrosive and toxic.
- Kimwipes (Kimberly-Clark, code 34120)
- L-Glutamine (US Biological, cat. no. G7120)
- Liquid nitrogen
 - ! **CAUTION** Liquid nitrogen is very cold, so handle it with care.
- Luer-Lok syringes, 5 ml (BD, cat. no. 309646)
- Metabolomics amino acid mix with 1.25–2.5 mM of 17 isotopically labeled amino acids (Cambridge Isotope Laboratories, item no. MSK-A2-1.2)
- Methanol, Optima LC/MS grade (Thermo Fisher Scientific, cat. no. A456-4)
 - ! **CAUTION** Methanol is a toxic and flammable liquid and vapor.
- Microcentrifuge tubes, 1.5 ml (Corning, prod. no. MCT-150-C)
- Microcentrifuge tubes, 2 ml (Sorenson BioScience, cat. no. 12000)
- Micro slides (VWR, cat. no. 48311-702)
- MitoTracker Deep Red FM (MTDR; Thermo Fisher Scientific, cat. no. M22426)
- Nail polish (Electron Microscopy Sciences, cat. no. 72180)
- Nonfat dry milk (LabScientific, cat. no. M-0842)
- Novex WedgeWell 12% Tris-glycine gels, 15 well (Thermo Fisher Scientific, cat. no. XP00125BOX)
- Paraformaldehyde (PFA) aqueous solution, 16% (wt/vol) solution (Electron Microscopy Sciences, cat. no. 15710)
 - ! **CAUTION** PFA is a very toxic and flammable liquid.
- Penicillin–streptomycin (Sigma-Aldrich, cat. no. P4333-100ml)
- Pierce ECL western blotting substrate (Thermo Fisher Scientific, cat. no. 32106)
- Phenol red, 0.5% (wt/vol) solution (Sigma-Aldrich, cat. no. P0290)
- Plasmids carrying the *gag*, *pol* genes (Addgene plasmid no. 8449)³⁵ and the *vsv-g* gene (Addgene plasmid no. 8454)³⁵. Plasmids carrying the *3XMyc-EGFP-OMP25* gene (Control-MITO construct; Addgene plasmid no. 83355)²¹ and *3XHA-EGFP-OMP25* gene (HA-MITO construct; Addgene plasmid no. 83356)²¹
 - ▲ **CRITICAL** The Control-MITO and HA-MITO constructs confer resistance to blasticidin ▲ **CRITICAL** Plasmid DNA is prepared from bacterial stabs using standard techniques and a miniprep kit (QIAGEN, cat. no. 27106).
- Polybrene infection/transfection reagent (EMD Millipore, cat. no. TR-1003-G)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405)
- Potassium phosphate monobasic (KH₂PO₄; Sigma-Aldrich, cat. no. P5655)
- Potassium hydroxide (KOH), 45% (wt/wt) solution (J.T. Baker, prod. no. 3143-01)
 - ! **CAUTION** KOH is corrosive and toxic.
- Protease inhibitor cocktail tablets (Roche, cat. no. 05 892 791 001)

- PVDF membranes, 0.45- μ m pores (Thermo Fisher Scientific, cat. no. IPVH00010)
- Sodium bicarbonate (US Biological, cat. no. S4000)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S9888)
- SDS pellets (Sigma-Aldrich, cat. no. 75746)
- Sodium hydroxide (NaOH), 50% (wt/wt) solution (J.T. Baker, prod. no. 3727-01) **! CAUTION** NaOH is corrosive and toxic.
- Sodium hydroxide pellets (NaOH pellets; AmericanBio, cat. no. AB01916-01000) **! CAUTION** NaOH pellets are corrosive and toxic.
- Sodium phosphate, dibasic (Na₂HPO₄; Sigma-Aldrich, cat. no. S5136)
- Tissue-culture dishes, 6 cm (Corning, prod. no. 430196)
- Tissue-culture dishes, 15 cm (Corning, prod. no. 353025)
- Tissue-culture plates, 6 well (Corning, prod. no. 3506)
- Tissue-culture plates, 24 well (Corning, prod. no. 3526)
- Tris (AmericanBio, cat. no. AB02000-05000)
- Tris hydrochloride (Tris-HCl; AmericanBio, cat. no. AB02005-05000)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Trypsin powder (Worthington Biochemical Corporation, cat. no. LS003704)
- Tween 20 (Thermo Fisher Scientific, cat. no. BP337-500)
- VectaShield hard-set antifade mounting medium with DAPI (Vector Laboratories, cat. no. H-1500)
- Water, Optima, LC/MS grade (Thermo Fisher Scientific, cat. no. W6-4)
- Western Lightning Plus-ECL (ECL plus; Perkin Elmer, cat. no. NEL103001EA)
- Wheaton glass bottles (Wheaton Science, part no. W216842) **▲ CRITICAL** These bottles do not need washing but should not have been used before.
- Wide-bore p1000 tips (Thermo Fisher Scientific, cat. no. 02-707-460)
- X-tremeGENE 9 DNA transfection reagent (Roche, cat. no. 06 365 779 001)

EQUIPMENT

- Vacuum aspirator
- Chromatography and mass spectrometry instruments (e.g., Thermo Fisher Scientific QExactive benchtop Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, and coupled to a Dionex UltiMate 3000 UPLC system with an EMD Millipore ZIC-pHILIC polymeric analytical column (2.1 \times 150 mm, 5- μ m particle size). The analytical software used is XCalibur Quanbrowser v2.2 or higher.)
- DynaMag spin magnet (Thermo Fisher Scientific, cat. no. 12320D)
- End-over-end rotator (e.g., ThermoLyne LabQuake rotator; Barnstead International, cat. no. 400110)
- Fiji software and plug-ins (<http://imagej.net/Fiji/Downloads>)³⁶
- Flatbed scanner
- Glassware for handling LC/MS-grade solvents, and for preparing LC/MS solutions (i.e., extraction solution, pHILIC buffer A), KPBS, and LC/MS PBS (e.g., Thermo Fisher Scientific borosilicate glass cylinders, beakers) **▲ CRITICAL** Glassware should be new and should be washed as indicated in the 'Reagent Setup' section.
- Heating block (e.g., analog dry block heater; VWR, cat. no. 12621-108)
- Homogenizer, 2-ml vessel (VWR, cat. no. 89026-386) **▲ CRITICAL** Wash the vessel with deionized water before and after use and in between samples. It is important to remove as much water as possible from the vessel by shaking it vigorously.
- Homogenizer, plain plunger (VWR, cat. no. 89026-398) **▲ CRITICAL** Wash the plunger with deionized water before and after use and in between samples. It is important to remove as much water as possible from the plunger by shaking it vigorously.
- ImageJ software and plug-ins (ImageJ, <https://imagej.nih.gov/ij/plugins/index.html>)^{37,38}
- Imaris software (Bitplane, <http://www.bitplane.com>)
- Microcentrifuge, refrigerated (e.g., Thermo Fisher Scientific Sorvall Legend Micro 17R microcentrifuge)
- Microcentrifuge (e.g., Thermo Fisher Scientific, model no. Eppendorf 5424 with model no. FA-45-24-11 rotor)
- Milli-Q water system (e.g., Milli-Q water purification system with Biopak Polisher)
- Minifuge, Galaxy Mini (VWR, model no. 93000-196)
- Orbiter (e.g., The Belly Dancer Shaker; IBI Scientific, model no. BDRAA115S)
- Owl VEP-2 mini tank electroblotting system (Thermo Fisher Scientific, cat. no. VEP-2)
- pH meter (e.g., Beckman Coulter, model no. pH1 510 pH meter equipped with a corresponding pH probe)
- Plate stirrer (e.g., hot plate stirrer; VWR, cat. no. 12365-382)

- PowerPac basic power supply (Bio-Rad, cat. no. 1645050)
- Stir bars (Thermo Fisher Scientific, cat. no. 22-067173)
- Tissue-culture centrifuge (e.g., Beckman Coulter, model no. Allegra X-12R with model no. SX4750A rotor and corresponding adaptors and plate carriers)
- Vortexer (e.g., Vortex-Genie 2; Scientific Industries, cat. no. SI-0236)
- XCell SureLock mini-cell electrophoresis system (Thermo Fisher Scientific, cat. no. EI0001)

REAGENT SETUP

▲ CRITICAL Deionized water is used for all solutions unless indicated otherwise.

▲ CRITICAL A dedicated set of brand-new glassware and equipment should be used to handle the LC/MS-grade solvents and to make the extraction solution and pHILIC buffer A. Brand-new glassware and equipment should be generously washed three times with LC/MS-grade water and then three times with LC/MS-grade methanol and left to dry at room temperature (21 °C) before use.

! CAUTION Methanol is highly flammable and toxic, so exercise caution.

▲ CRITICAL A dedicated set of reagents (e.g., KCl, KOH), brand-new glassware, and equipment should be used to prepare the KPBS and LC/MS PBS. Brand-new stir bars, glassware, and pH meter probes should be generously washed three times with Milli-Q water and left to dry at room temperature before use. The aforementioned pieces of equipment should be thoroughly washed with Milli-Q water after each preparation of KPBS or LC/MS PBS. Using Milli-Q water here instead of LC/MS-grade water helps reduce costs and does not seem to have a major effect on downstream analyses.

Base DMEM Dissolve 135 g of DMEM powder, 37 g of sodium bicarbonate, and 3 g of L-glutamine in 10 liters of Milli-Q water. Adjust the pH to 7 using HCl, filter into sterile, autoclaved glass bottles using a 0.22- μ m filter-top, and store the bottles at 4 °C. These solutions are stable for at least 1 month.

! CAUTION HCl is highly corrosive and toxic, so handle it with care.

Penicillin–streptomycin Add 100 ml of stock solution to 100 ml of Milli-Q water, prepare aliquots, and store them at –20 °C. These aliquots are stable for at least 1 month.

Complete DMEM Complete DMEM is base DMEM supplemented with 10% (vol/vol) IFS and 1% (vol/vol) penicillin–streptomycin. Add 50 ml of IFS and 5 ml of penicillin–streptomycin to 450 ml of base DMEM. Filter into a sterile, autoclaved glass bottle using a 0.22- μ m filter-top and store it at 4 °C. This solution is stable for at least 1 month.

PBS This version of PBS is for routine tissue culture and is 137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, and 1.47 mM KH₂PO₄, pH 7.1, in Milli-Q water. Dissolve 8 g of NaCl, 1.14 g of Na₂HPO₄, 200 mg of KCl, and 200 mg of KH₂PO₄ in 800 ml of Milli-Q water. Adjust the pH to 7.1 with HCl and bring the volume up to 1 liter with additional Milli-Q water. Autoclave in a glass bottle to sterilize. This buffer can be stored at room temperature for at least several months.

Trypsin The trypsin solution is 0.15% (wt/vol) trypsin, 2.4 mM EDTA, and 0.0005% (wt/vol) phenol red. Dissolve 1.5 g of trypsin powder in 750 ml of sterile PBS, and add 4.8 ml of EDTA and 1 ml of phenol red (0.5% (wt/vol) solution). Bring the volume up to 1 liter with additional sterile PBS and adjust the pH to 7 with HCl. Filter with a 0.22- μ m filter-top, prepare aliquots, and store them at –20 °C. These aliquots are stable for at least 1 month.

! CAUTION EDTA is toxic, so handle it with care.

pHILIC buffer A pHILIC buffer A is 20 mM ammonium carbonate and 0.1% (wt/vol) ammonium hydroxide. Dissolve 0.481 g of ammonium carbonate in 240 ml of LC/MS-grade water. Add 892 μ l of ammonium hydroxide (28% (wt/vol) solution) and adjust to a final volume of 250 ml with LC/MS-grade water. This buffer can be stored at room temperature for 1 week. **! CAUTION** Ammonium hydroxide is corrosive, so handle it with care.

Extraction solution This solution is 80% (vol/vol) LC/MS-grade methanol, 20% (vol/vol) LC/MS-grade water, and 250–500 nM of 17 internal standards. Combine 160 ml of LC/MS-grade methanol with 40 ml of LC/MS-grade water and 40 μ l of the metabolomics mix containing 17 isotopically labeled amino acids. Divide the solution into aliquots in Wheaton glass bottles and store them at –20 °C. This solution is stable for at least several months.

KPBS KPBS is 136 mM KCl and 10 mM KH₂PO₄, pH 7.25. This LC/MS-compatible buffer is designed to approximate the pH and abundance of potassium within the cytosol, minimize the exposure of isolated mitochondria to excess sodium, and maintain mitochondrial integrity. Dissolve 5.07 g of KCl and 0.68 g of KH₂PO₄ in 450 ml of LC/MS-grade water. Adjust the

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pH to 7.25 using KOH and bring the volume up to 500 ml with LC/MS-grade water. Filter it through a 0.22- μ m filter-top bottle and store it at 4 °C. KPBS is stable for months. **! CAUTION** KOH is corrosive and toxic, so handle it with care.

LC/MS PBS This version of PBS is for washing cells before mitochondrial immunopurification and is 137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, and 1.47 mM KH₂PO₄, pH 7.1, in LC/MS-grade water. Dissolve 8 g of NaCl, 1.14 g of Na₂HPO₄, 200 mg of KCl, and 200 mg of KH₂PO₄ in 800 ml of LC/MS-grade water. Adjust the pH to 7.1 and bring the volume up to 1 liter with additional LC/MS-grade water. Filter through a 0.22- μ m filter-top bottle and store it at 4 °C. LC/MS PBS is stable for months.

1 M Tris-HCl, pH 7.4 Dissolve 12.1 g of Tris in 70 ml of water. Adjust the pH to 7.4 using HCl and bring the volume up to 100 ml. Store it at 4 °C. The solution is stable for at least 1 year.

5 M NaCl Dissolve 29.2 g of NaCl in 80 ml of water and then bring the volume up to 100 ml. Store it at room temperature. The solution is stable for years.

20% (vol/vol) Triton X-100 Add 20 ml of Triton X-100 to 80 ml of water. Store the solution at 4 °C. The detergent is stable for at least 1 year.

Triton X-100 lysis buffer This detergent lysis buffer is 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, and protease inhibitor. Add 5 ml of 1 M Tris-HCl, pH 7.4, 3 ml of 5 M NaCl, 200 μ l of 500 mM EDTA, pH 8.0, and 5 ml of 20% (vol/vol) Triton X-100, and bring up the volume to 100 ml with water. Store it at 4 °C. The solution is stable for years. Grind up and dissolve 1 tablet of protease inhibitor in 10 ml of this solution; note that there is always some residual solid material that does not fully dissolve. Prepare aliquots and store the resulting lysis buffer at -20 °C. These aliquots are stable for at least 1 month, but avoid multiple freeze-thaw cycles.

5 \times Laemmli sample buffer (5 \times LB) This buffer is 237 mM Tris-HCl, pH 6.8, 10% (wt/vol) SDS, 25% (vol/vol) 2-mercaptoethanol, 25% (vol/vol)

glycerol, and 0.056% (wt/vol) bromophenol blue. Dissolve 1.68 g of Tris-HCl powder and 4.5 g of SDS in 22.5 ml of water. Add 11.25 ml of 2-mercaptoethanol and 11.25 ml of glycerol, and adjust the pH to 6.8 with NaOH first, and then add 0.025 g of bromophenol blue. Pass the solution through a 0.45- μ m filter, prepare aliquots, and store them at -20 °C. These aliquots are stable for at least several months. **! CAUTION** NaOH is corrosive and toxic, so handle it with care.

10 \times TBST This buffer is 1.37 M NaCl, 200 mM Tris-HCl, pH 7.6, and 1% (vol/vol) Tween 20. Dissolve 160 g of NaCl, 50.1 g of Tris-HCl powder, and 9.91 g of Tris in 1.7 liters of water. Add 20 ml of Tween 20, adjust the pH to 7.6, and bring the volume up to 2 liters with water; store the solution at room temperature. The 10 \times buffer is stable for at least several months. Dilute to 1 \times with water before use.

10 \times SDS running buffer This buffer is 248 mM Tris, 1.92 M glycine, and 1% (wt/vol) SDS. Dissolve 30 g of Tris, 144 g of glycine, and 10 g of SDS in 1 liter of water. Store the solution at room temperature. The 10 \times buffer is stable for at least several months. Dilute to 1 \times with water before use.

Transfer buffer This buffer is 10 mM CAPS and 10% (vol/vol) ethanol. Add 200 ml of 190 proof ethanol to 1.8 liters of water. Dissolve 4.42 g of CAPS and 8 NaOH pellets. This buffer is best when made fresh. **! CAUTION** Ethanol is flammable, so exercise caution. NaOH pellets are corrosive and toxic, so handle them with care.

5% (wt/vol) milk Dissolve 20 g of nonfat dry milk in 400 ml of 1 \times TBST. Store the solution at 4 °C. The milk is usable only for 2 weeks.

4% (wt/vol) PFA Add 9 ml of 16% (wt/vol) PFA to 27 ml of PBS. Prepare aliquots and store them at -20 °C. The solution is stable for at least several months, but avoid multiple freeze-thaw cycles. **! CAUTION** PFA is toxic and flammable, so handle it with care.

PROCEDURE

Generation and validation of mammalian cells with epitope-tagged mitochondria ● TIMING 11–15 d

1| Plate 700,000 HEK293T cells in 4 ml of complete DMEM in a 6-cm dish for each construct, and let the cells attach overnight.

▲ **CRITICAL STEP** HEK293T cells from different labs vary in their ability to produce high-titer virus and can be sensitive to conditions such as overcrowding and inadequate media changing. In addition, HEK293T cells can be sensitive to 0.15% (wt/vol) trypsin, so it is important to remove residual trypsin from the cell suspension by centrifugation and resuspension in fresh medium when plating.

2| For each plate of HEK293T cells, assemble the mixture described below in 200 μ l of base DMEM.

Component	Amount per 6-cm dish
<i>gag-pol</i> construct	1 μ g
<i>vsv-g</i> construct	0.1 μ g
Control-MITO or HA-MITO constructs	1 μ g
X-tremeGENE 9	6 μ l

3| Vortex each tube gently for 6 s and incubate for 30 min at room temperature. During this time, carefully replace the medium of each plate of HEK293T cells with 4 ml of fresh complete DMEM.

4| Add the mixtures from Step 2 dropwise around the plate, gently swirl to mix, and incubate cells for 18–24 h.

5| Replace the medium of each plate with 4 ml of fresh complete DMEM and wait for 24 h.

6| Collect the medium while trying to minimize contamination with HEK293T cells and then pass through a 0.45- μ m Acrodisc filter using a syringe. The virus can be stored on ice for immediate use or at -80 °C.

■ **PAUSE POINT** The virus can be stored at -80 °C for years. Because viruses are sensitive to freeze-thaw cycles, we recommend making multiple aliquots that do not undergo more than two freeze-thaw cycles.

7| Because virally infected cells will be selected with blasticidin and different cell lines have varying sensitivities to this agent, it is important to empirically determine the working concentration needed to completely kill uninfected cells. To do this, treat 100,000 cells (for example, HeLa cells) in 3 ml of medium per well of a 6-well plate with a series of different blasticidin media concentrations (0 µg/ml, 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml) and examine cells after 4 d of treatment.

8| To introduce the Control-MITO or HA-MITO constructs into the desired cell lines, infect 50,000 cells in 2 ml of medium containing 4 µg/ml polybrene per well of a 6-well plate. Leave one well with uninfected cells to serve as a control for selection with blasticidin later on. The amount of virus needed per infection is dependent on the titer of the virus and the infectability of the cell line being used, but generally ranges from 2.5 to 50 µl.

9| Centrifuge the plate at ~1,100g for 1 h at 37 °C and then incubate cells with the virus overnight.

10| Remove the virus from the infected cells, replace with 3 ml of medium containing the appropriate concentration of blasticidin as determined in Step 7, and select with blasticidin for 4 d. By the end of 4 d, there should be complete killing of the uninfected cells from Step 8. For HeLa cells, we typically select with a working concentration of 8.5 µg/ml blasticidin.

11| Use fluorescence-activated cell sorting (FACS) to obtain cells with an appropriate amount of EGFP signal, making sure to include uninfected wild-type cells in the FACS analysis for establishing gates. Note that although sorting cells twice can increase the purity of the target cell population, the final distribution of EGFP signals in a sorted population can still extend beyond the borders of a given sorting window. In terms of the optimal amount of EGFP signal (i.e., the amount of EGFP signal that maximizes the yield and purity of mitochondrial capture), this can vary between cell lines and should be determined using immunoblot analysis (see Steps 51–58). We have found that lower expression of the constructs is generally better, achieving amounts of mitochondrial capture similar to those of higher-expressing systems while substantially reducing contamination with organelles such as the peroxisome (Fig. 2; Supplementary Fig. 1). It is important to note that mitochondria can directly interact with certain organelles, such as the endoplasmic reticulum and peroxisomes, in living cells^{32,39}; the strength of these interactions may vary among cell types and lead to different amounts of contamination in immunopurified mitochondria, but there should generally be enrichment of mitochondrial markers in the IP lysates as compared with whole-cell lysates. Cells should also be visually inspected to ensure that there is proper colocalization of EGFP with the mitochondrial dye, MTRD, and that mitochondria appear morphologically normal (see Steps 61–68).

▲ CRITICAL STEP Proper generation of mammalian cells expressing the Control-MITO construct (Control-MITO cells) or HA-MITO construct (HA-MITO cells) is critical for optimal immunocapture of mitochondria.

12| For future use, freeze multiple aliquots of Control-MITO and HA-MITO cells in cryogenic vials using freshly made, chilled growth medium containing 10% (vol/vol) DMSO. Cells should be stored at –80 °C for at least 1 d before transferring to long-term storage in liquid nitrogen. Although the pMXs-retroviral vector system generally provides stable expression across multiple cell lines that have been tested, it is worthwhile to have frozen original stocks to return to should the constructs become silenced.

! CAUTION Liquid nitrogen is very cold, so exercise caution.

■ PAUSE POINT Cells are viable in liquid nitrogen for years.

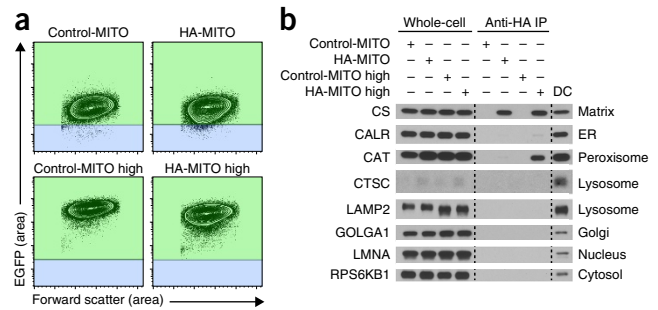


Figure 2 | The degree of HA-MITO construct expression affects the purity of isolated mitochondria. **(a)** Illustrative flow cytometric plots of HeLa cells with appropriate expression levels of the Control-MITO construct (Control-MITO) and the HA-MITO construct (HA-MITO) versus those with high expression levels of the Control-MITO construct (Control-MITO high) and the HA-MITO construct (HA-MITO high). The light green and light blue quadrants denote areas of EGFP-positivity and background EGFP signal, respectively. The gating strategy is detailed in **Supplementary Figure 1**. The forward scatter (area) axis is linear and the EGFP (area) axis is logarithmic. The percentage of single, viable cells with EGFP signal above background was greater than 98.5% for all cell lines analyzed. For each sample, 100,000 cells were analyzed on a FACSAria IIU SORP sorter (BD Biosciences) using the FACSDiva software (BD Biosciences) and the data were converted into contour plots with outliers using the FlowJo software (FlowJo). **(b)** Mitochondria isolated from HA-MITO cells have greater purity than mitochondria isolated from HA-MITO high cells and mitochondria isolated using an abbreviated form of differential centrifugation described previously²¹. SDS-PAGE and immunoblotting were used to interrogate whole cells and mitochondria isolated via rapid differential centrifugation (DC) or an anti-HA-IP using HA-MITO or HA-MITO high cells. The name of the protein marker used appears to the left of each blot and the corresponding subcellular compartment appears on the right. The dotted lines indicate where different lanes of the same membrane are brought together. These results are representative of three experiments. For this figure, HeLa cells were cultured in complete DMEM, authenticated by the Duke University DNA Analysis Facility, and tested for mycoplasma contamination. ER, endoplasmic reticulum; Golgi, Golgi complex; Matrix, mitochondrial matrix.

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13| To recover cells from frozen storage, quickly thaw the cells in a 37 °C water bath, gently resuspend the cells by pipetting, mix with 10 ml of growth medium, centrifuge the cells at 524*g* for 5 min at room temperature, aspirate the supernatant, and plate the cells with appropriate amounts of growth medium.

Isolation and processing of mitochondria ● **TIMING 1–2 h per sample; Steps 23–32 take ~12 min**

▲ **CRITICAL** Perform Steps 14 and 15 with the samples, magnet, and solutions on ice.

▲ **CRITICAL** To increase the speed of the workflow, process one plate of Control-MITO or HA-MITO cells completely (i.e., perform Steps 17–37) before processing another plate of cells.

14| Resuspend anti-HA beads by shaking the bottle until there is a homogeneous suspension. Prepare 200- μ l aliquots of anti-HA beads in a separate 1.5-ml tube for each IP using Control-MITO or HA-MITO cells.

15| Collect the beads on a magnet and carefully aspirate the overlying solution using a gel-loading tip attached to a vacuum aspirator by applying the pipette tip to the side of the 1.5-ml tube farthest from the magnet and gently proceeding to the bottom of the tube. Add 1 ml of ice-cold KPBS to each tube and resuspend by inversion until no more aggregates are present on the bottom of the tube and the suspension is homogeneous. Residual material left on the cap can be brought down with a pulse-spin (<1 s) in a minifuge. Perform this step three times and leave the bead suspension on ice when done.

16| Move to the cold (4 °C) room so that Steps 17–37 can be performed at a suitable temperature.

▲ **CRITICAL STEP** Because of the relatively labile nature of metabolites, it is important to move quickly and extract metabolites as soon as possible, especially once cells have been homogenized. Perform all of Steps 17–37 in the cold room on ice until metabolite extraction and detergent lysis have been completed. Prechill the vessel, plunger, centrifuge, end-over-end rotator, minifuge, tubes, pipette tips, solutions, and cell scrapers. All centrifugation steps should be carried out at 4 °C.

17| Starting with ~30 million adherent cells in a 15-cm plate, quickly pour off the medium and aspirate any residual liquid. Note that although this protocol has been designed for adherent cells, it can in theory be adapted for suspension cells as well.

18| Pour 30 ml of premeasured, ice-cold LC/MS PBS onto the cells and wash them by swirling the liquid around the plate and then rocking it back and forth several times. Pour off the LC/MS PBS and thoroughly aspirate any residual liquid. Perform this step twice.

19| Add 1 ml of KPBS to the plate and quickly scrape the cells into the KPBS with the scraper's nonangled face oriented away from the cells. To avoid unnecessary stress on the cells, harvest the cells by scraping down the plate in one motion, rather than moving back and forth.

20| Transfer the cell suspension to a 2-ml tube and centrifuge at 1,000*g* for 2 min at 4 °C.

▲ **CRITICAL STEP** When processing samples for immunocapture of mitochondria, handle cells and organelles using wide-bore p1000 tips to decrease the chances of damaging them.

21| Remove as much supernatant as possible without disturbing the pellet, and resuspend well with 1 ml of KPBS and gentle pipetting to obtain a homogeneous single-cell suspension. Transfer the suspension to the homogenizer vessel.

22| Homogenize cells with 25 strokes of the plunger.

▲ **CRITICAL STEP** Note that the plunger and vessel should form a reasonably snug fit with one another, or else there will be insufficient homogenization of the sample. Each stroke should bring the tip of the plunger to the bottom of the vessel and then up to the highest level where no air bubbles form. Each stroke should be a smooth, continuous motion with no rotation of the plunger and should require ~1.5–2 s to complete. Avoid forming and trapping air bubbles, as these can damage organelles. Note that this manner of homogenization prioritizes the integrity of organelles at the expense of yield, so a substantial proportion of cells are not sufficiently ruptured to liberate all of their mitochondria. Although we have found 25 strokes without rotation of the plunger to be a good starting point for homogenizing cells, the optimal manner of homogenization (e.g., number of strokes, number of rotations of the plunger) can vary between cell lines and should be determined before attempting mitochondrial isolation (**Box 1; Supplementary Fig. 2**). Rotating the plunger while delivering strokes provides stronger homogenization and is typically accomplished using an overhead stirrer with matching plunger and vessel, as described previously⁴⁰.

Box 1 | Optimization of cellular homogenization ● TIMING 1.5–10 h, depending on the number of homogenization conditions examined

Determining the minimum amount of homogenization necessary for sufficient cell rupture is critical to successfully profiling mitochondrial metabolites. Poor homogenization leads to inadequate amounts of mitochondria being available for immunocapture. However, excessive homogenization can damage mitochondria liberated from cells, leading to leakage of matrix metabolites.

1. Before processing cells, add 985 μ l of KPBS to the desired number of 1.5-ml tubes and leave these tubes on ice. One 1.5-ml tube is needed for sampling unhomogenized cells and one is also needed for each successive homogenization condition.
2. Process HA-MITO cells using Steps 16–20 of the PROCEDURE (i.e., as if one were using these cells for the actual workflow) but do not prepare any tubes or reagents for performing an IP, metabolite extraction, or detergent lysis, as those steps are not needed for optimizing the homogenization of the cells at this stage.
3. Following Step 20 of the PROCEDURE, remove as much supernatant as possible without disturbing the pellet, and resuspend well with 1 ml of KPBS and gentle pipetting to get a homogeneous single-cell suspension.
4. Gently mix 15 μ l of the cell suspension with 985 μ l of KPBS and leave this 1-ml solution of diluted cells on ice for later use. This sample contains nonhomogenized cells.
5. Transfer the remaining cell suspension to the homogenizer and apply increasing amounts of homogenization, making sure to gently mix 15 μ l of the homogenate with 985 μ l of KPBS and keeping this 1-ml solution of diluted cells on ice after each homogenization condition. As mentioned in Step 22 of the PROCEDURE, 25 strokes without rotation of the plunger is a good starting point for homogenizing cells. Increasing the number of nonrotating strokes by 10 is also a reasonable approach to progressively increase the amount of homogenization.
6. Transfer each 1-ml solution of diluted cells to a well of a 6-well plate, gently rock the plate back and forth to evenly distribute the contents, and visualize the cells by microscopy. On the basis of our experience and the existing literature³⁰, microscopic examination of a homogenate of HA-MITO cells should primarily reveal several different types of objects: cells (e.g., EGFP-positive objects with various morphologies, granular interiors, and occasionally membrane blebs), free nuclei (e.g., EGFP-negative, smooth ovals/circles with minimal internal granularity, no membrane blebs, and prominent nucleoli that appear as dark dots), and free organelles (e.g., black dots that are smaller than cells and free nuclei). Examples of these objects are shown in **Supplementary Figure 2**.
7. To assess the degree of homogenization in a sample, calculate the ratio of cells to free nuclei. This can be done by taking representative micrographs while inspecting one entire row of the well. Count the number of cells and free nuclei in the resulting micrographs (see descriptions of these objects above) using Fiji and the Cell Counter plugin, and calculate the ratio of total cells to total free nuclei. We typically examine enough images to accrue 500–1,000 cells per sample for these analyses. For HA-MITO HeLa cells, we have found that 25 strokes without rotation of the plunger results in homogenates with roughly 20–40 cells for each free nucleus and enables successful profiling of mitochondrial metabolites. As such, the minimal amount of homogenization that can yield a similar ratio of cells to free nuclei is generally a desirable point to begin with when homogenizing a new cell line for mitochondrial metabolomics. However, further adjustments may be warranted, should the metabolite profiling results appear suboptimal.

23| Pour the homogenate into a new 1.5-ml tube and centrifuge at 1,000g for 2 min at 4 °C. During the centrifugation, invert a tube of prewashed anti-HA beads until the suspension is homogeneous and free of aggregates. Pulse-spin (<1 s) to collect any residual beads on the lid of the tube.

24| Collect the beads with the magnet and aspirate the overlying solution as described in Step 15. The supernatant from the centrifuged sample in Step 23 is the postnuclear supernatant (PNS). Quickly collect the PNS while avoiding the pellet, and apply the solution to the beads. We typically leave ~100 μ l of PNS to avoid disturbing the pellet.

25| Resuspend the beads using the PNS by gentle pipetting until there are no more beads present on the side of the 1.5-ml tube and the entire mixture is homogeneous.

26| Incubate the mixture on an end-over-end rotator for 3.5 min.

27| Pulse-spin (<1 s) to remove any residual material on the lid of the 1.5-ml tube.

28| Collect the beads on the magnet and thoroughly aspirate the overlying solution as described in Step 15. Collect the beads with 1 ml of KPBS by pipetting to the side of the 1.5-ml tube several times. To help maintain the integrity of captured mitochondria, avoid pipetting KPBS directly onto the beads. To wash the beads, gently pipette up and down five times. The solution should be homogeneous with no visible aggregates. Perform this step three times.

29| Transfer the 1-ml suspension of beads to a new 1.5-ml tube. This can help reduce background during metabolite extraction and detergent lysis.

▲ CRITICAL STEP If you are performing the IP purely for immunoblot analysis, proceed directly to Step 34 with the 1-ml suspension of beads.

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30| Transfer 250 μl of the suspension of beads to a new 1.5-ml tube and set this tube aside for now. This sample will be for detergent lysis.

31| For the remaining suspension of beads, collect the beads on a magnet and aspirate the overlying solution as described in Step 15.

32| Extract the metabolites with 50 μl of extraction solution by first pipetting the solution along the side of the 1.5-ml tube so that the solvent runs over the beads. Repeat this step four times using the same 50 μl of extraction solution.

33| Because the beads may still be attached to the side of the 1.5-ml tube at this point, collect the beads and bring them to the bottom of the tube using extraction solution and the tip of the pipette. Pipette the extraction solution and beads up and down until homogeneous, and incubate on ice for 5 min.

▲ CRITICAL STEP To extract lipids and more nonpolar metabolites, use alternative reagents, such as chloroform.

34| Process the remaining 250- μl suspension of beads from Step 30 by aspirating and pipetting as described in Steps 31–33, but use 50 μl of Triton X-100 lysis buffer in place of the extraction solution and incubate on ice for 10 min after pipetting to homogeneity.

▲ CRITICAL STEP If you are performing the IP purely for immunoblot analysis, process the 1-ml suspension of beads from Step 29 by aspirating and pipetting as described in Steps 31–33, but use 100 μl of Triton X-100 lysis buffer in place of the extraction solution and incubate on ice for 10 min after pipetting to homogeneity. In general, avoid forming air bubbles in the lysate.

35| Once the metabolite extraction (i.e., the sample incubated with the extraction solution) or detergent lysis (i.e., the sample incubated with Triton X-100 lysis buffer) has been completed, collect the beads on a magnet and transfer the overlying solution to a new 1.5-ml tube using a gel-loading tip. Centrifuge both the metabolite extract and the detergent lysate at 17,000*g* for 10 min at 4 °C.

▲ CRITICAL STEP The resulting pellet may not be readily visible, so monitor the orientation of the tubes in the centrifuge.

36| Transfer the supernatant from the metabolite extract to a new 1.5-ml tube without disturbing the pellet, and store it at –80 °C.

■ PAUSE POINT The stability of metabolites at –80 °C can be quite variable, and so we recommend analyzing metabolites as soon as possible, ideally within several days following extraction.

37| For the detergent lysate, add 40 μl of the supernatant to 10 μl of 5 \times LB, vortex to mix, incubate at 100 °C for 5 min, pulse-spin to collect material on the lid, and store it at –20 °C.

■ PAUSE POINT Samples can be stored at –20 °C for up to a year.

Absolute quantification of metabolites by LC/MS ● TIMING 1–2 d

▲ CRITICAL We have provided the details for our LC/MS workflow below as a guide, but any LC/MS method with sufficient sensitivity, reproducibility, and linearity for the metabolites of interest can be used. Note that our methodology is optimized for analyzing polar metabolites, but in theory it can be modified for studying lipids and other nonpolar molecules.

It is important to note that for certain experiments relative rather than absolute quantification of metabolites may be sufficient, in which case it is not necessary to include standard curves with every LC/MS run and perform confocal microscopy and volumetric analysis for each experimental condition, thus shortening the workflow considerably.

38| For each metabolite to be analyzed using relative quantification or absolute quantification, first run authentic standards on the LC/MS instrument and confirm that they produce a robust peak at the correct *m/z* ratio. Document the retention time of the correct molecule. Ideally, each standard should be run on multiple days to determine the reproducibility and variability of the instrument. When determining which metabolites to interrogate, see our prior work for a list of metabolites involved in mitochondrial metabolism²¹.

39| For absolute quantification of metabolites, analyze standard curves in the same run as the experimental samples. To facilitate this analysis, assemble pools of metabolite standards by making 1 mM stocks of each metabolite standard in an appropriate LC/MS-grade solvent and store the stocks at –80 °C. On the day of an LC/MS run, use the 1 mM stocks and extraction solution to prepare fresh standard curves at the following concentrations: 1, 10, 100 nM, 1, 10, and 30 μM .

▲ CRITICAL STEP Because there is always residual KPBS when metabolites are extracted from beads, it is important to correct for LC/MS matrix effects resulting from the behavior of metabolites in the presence of KPBS. As such, we recommend comparing the behavior of standard curves generated with extraction solution and those made using extraction solution with

KPBS spiked in. We typically add KPBS at a 1/25 dilution and feel that this generally recapitulates the residual wet volume before extraction of an IP.

40| Using a Dionex UltiMate 3000 UPLC system, inject 5 μ l of each sample onto a ZIC-pHILIC 2.1 \times 150 mm (5- μ m particle size) column. No dilutions or modifications are made to the sample after extraction from the beads and before injection onto the LC/MS instrument. For each run, also inject 5 μ l of a sample containing 5 μ l of KPBS mixed with 50 μ l of extraction solution; this sample will be used to assess the purity of the KPBS and should generally have low to undetectable metabolite signal as compared with those of IP extracts. We have noticed, however, that citrate is present to some degree in KPBS, which can make analysis of this metabolite difficult at times. Despite trying different preparations and lot numbers of the components used in KPBS, we currently have not been able to eliminate this background and feel that citrate is likely a trace contaminant of the salts used in the solution.

41| Run the chromatographic gradient at a rate of 0.150 ml/min as follows: from 0–20 min, a linear gradient from 20% pHILIC buffer A, 80% acetonitrile to 80% pHILIC buffer A, 20% acetonitrile; from 20–20.5 min, a linear gradient from 80% pHILIC buffer A, 20% acetonitrile to 20% pHILIC buffer A, 80% acetonitrile; from 20.5–28 min, hold at 80% acetonitrile.

! CAUTION Acetonitrile is highly flammable, so exercise caution.

42| Introduce the column effluent directly into a QExactive benchtop Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe. Operate the mass spectrometer in full-scan, polarity-switching mode with the spray voltage set to \pm 3.0 kV, the heated capillary set to 275 $^{\circ}$ C, and the HESI probe set to 350 $^{\circ}$ C. Set the sheath gas flow to 40 units, the auxiliary gas flow to 15 units, and the sweep gas flow to 1 unit.

43| Acquire mass spectrometry data between 70 and 1,000 m/z , with the resolution set at 70,000, the AGC target at 10^6 , and the maximum injection time at 80 ms.

44| To increase the sensitivity of the instrument for metabolites that have absent or low signals, consider performing tSIM scans. For tSIM scans, the mass spectrometer is run using either positive or negative polarity. The optimal polarity is the one that gives the greatest signal for the authentic standard. For examples of instrument settings and acquisition parameters for tSIM scans, see our prior work²¹.

45| Identify and quantify metabolites using XCalibur QuanBrowser version 2.2 or higher. Set the mass accuracy window to 10 p.p.m. and the retention time window to 0.5 min. Use authentic standards to identify and quantify metabolites within samples. Within-batch mass deviation is typically less than 0.0005 Da, and retention time deviation is usually less than 0.25 min.

46| In each sample, calculate the relative abundance by dividing the raw peak area for each metabolite by the raw peak area of the relevant isotopically labeled internal standard. Ideally, each metabolite should be normalized to its own isotopically labeled equivalent, but this can be prohibitively expensive for large numbers of metabolites. In such cases, the peak areas can be normalized to either isotopically labeled phenylalanine or valine, whichever is closest in chemical structure and/or retention time to the analyte of interest (**Supplementary Data 2**).

47| For each dilution series of a metabolite standard, find the curve of best fit for the relative abundances as a function of metabolite concentration (e.g., ideally, $r^2 > 0.99$ and there is less than 20% deviation between calculated versus observed concentrations at each individual point in the curve). In general, we have found that a quadratic log–log equation provides the best fit for the broad range of metabolite concentrations used in this workflow.

48| Use the calibration curves from Step 47 to determine the concentrations of metabolites within the samples. For undetectable metabolites, assign concentrations of 0. If any metabolites have notable LC/MS matrix effects from KPBS as determined in Step 39, correct for them as well. In general, good-quality data sets should have very low or undetectable levels of fructose 1,6-bisphosphate and cystine in the HA-MITO IP samples, as these are metabolites predominantly found in the cytosol and lysosome, respectively.

49| For a metabolite to be considered mitochondrial, the abundance in the HA-MITO IP must be at least 1.5-fold greater than that in the Control-MITO IP in each of the three biological replicate sets. Exclude any metabolites that do not meet this criterion.

? TROUBLESHOOTING

PROTOCOL

50 | For metabolites that meet the criterion described in Step 49, calculate the 'matrix metabolite abundance' needed for Step 78 using the equations below, where units are given in parentheses:

$$\text{IP abundance} \left(\frac{\text{pmol}}{\text{IP}} \right) = \text{Metabolite extract concentration} (\mu\text{M}) \times \text{Metabolite extract volume} \left(\frac{\mu\text{L}}{\text{IP}} \right)$$

$$\text{Matrix metabolite abundance} \left(\frac{\text{pmol}}{\text{IP}} \right) = \text{IP abundance}_{\text{HA-MITO}} - \text{IP abundance}_{\text{Control-MITO}}$$

Immunoblot analysis of the IP material ● TIMING 2–3 d

▲ **CRITICAL** Immunoblot analysis serves two main purposes in this workflow: measuring the whole-cell equivalents of isolated mitochondria to facilitate comparisons between experimental samples and allow for absolute quantification of matrix metabolites, and assessing the yield and purity of isolated mitochondria when optimizing immunocapture for a new cell line (see Steps 1–11). Whole-cell lysates are useful here as additional samples for analysis, as they allow one to assess the relative enrichment and depletion of subcellular compartments during the immunocapture process. Note that even if relative quantification—rather than absolute quantification—is being performed, immunoblot analysis is still necessary to account for different amounts of isolated mitochondria across HA-MITO IP samples.

51 | Lyse the desired number of cells in Triton X-100 lysis buffer, mix well, incubate on ice for 10 min, centrifuge at 17,000*g* for 10 min at 4 °C, and add the appropriate amount of 5× LB. It is best to use cells that have experienced the same culture conditions (e.g., treatment with a drug) as those used for mitochondrial immunocapture to achieve experimental consistency.

▲ **CRITICAL STEP** When choosing the amount of cells and volume of lysis buffer for looking at subcellular markers, aim for roughly 30,000–120,000 cell equivalents per 15 μL, as this will generally result in good signal for most interrogated proteins, although different cell lines may require additional optimization. For measuring whole-cell equivalents of isolated mitochondria, run a standard curve composed of ~80,000 cell equivalents per 15 μL and two successive 2-fold dilutions. Using citrate synthase (CS) as a marker, measure the whole-cell equivalents of isolated mitochondria using this standard curve. If the CS signals of the IP samples do not lie within the standard curve, adjust accordingly. We have found CS to be a good marker because it is predominantly localized to the mitochondrial matrix within cells²⁴. In addition, its solubility⁴¹ and residence within the matrix²⁴ make CS a good proxy for mitochondria with intact inner membranes.

52 | Load 15 μL of IP lysates and whole-cell lysates from Steps 37 and 51, respectively, on a 12% Tris-glycine gel and resolve by SDS-PAGE at 100 mV.

53 | Transfer for 2.5 h at 50 V onto a 0.45-μm PVDF membrane.

54 | Block the membrane in 5% (wt/vol) milk for 45 min at room temperature.

55 | Incubate the membrane for 24–48 h with the desired primary antibodies at 4 °C. For quantifying whole-cell equivalents in the IP material, only CS must be interrogated. For examining the purity of immunocapture, probe for markers of multiple subcellular compartments. Suitable antibodies to use are detailed in **Table 1**.

56 | Wash the membrane three times with TBST, for 5 min each time.

57 | Incubate the membrane with the appropriate secondary antibody (diluted 1/5,000 in 5% (wt/vol) milk) for 1 h at room temperature, repeat Step 56, and visualize with ECL and film. For visualizing CTSC, ECL Plus can be useful.

58 | Scan desired films and save as 16-bit images.

59 | Use ImageJ to perform densitometry analysis on scanned films. Using the standard curve of whole-cell lysates, generate a best-fit line (typically, $r^2 > 0.97$), and determine the number of whole-cell equivalents present in each of the HA-MITO IP lysates. There is usually undetectable CS signal in the Control-MITO IP lysates.

60 | To calculate the number of equivalents present in the IP sample used for metabolite extraction, multiply by three because the amount of beads used for metabolite extraction is three times that used for detergent lysis. The resulting number is the 'mitochondrial yield' needed for Step 78.

TABLE 1 | Protein markers for subcellular compartments and the corresponding antibodies.

Protein	Apparent molecular weight (kDa)	Subcellular compartment	Reagent	Dilution
CS	45	Mitochondrial matrix	CST anti-CS antibody (cat. no. 14309)	1/1,000
RPS6KB1	50–80	Cytosol	CST anti-RPS6KB1 antibody (cat. no. 2708)	1/1,000
CALR	60	Endoplasmic reticulum	CST anti-CALR antibody (cat. no. 12238)	1/1,000
GOLGA1	90	Golgi complex	CST anti-GOLGA1 antibody (cat. no. 13192)	1/1,000
LAMP2	100–140	Lysosome	SCBT anti-LAMP2 antibody (cat. no. sc-18822)	1/1,000
CTSC	15–20 (mature form)	Lysosome	SCBT anti-CTSC antibody (cat. no. sc-74590)	1/500
LMNA	75	Nucleus	SCBT anti-LMNA antibody (cat. no. sc-20680)	1/1,000
CAT	60	Peroxisome	CST anti-CAT antibody (cat. no. 12980)	1/1,000

Note: We have found that catalase (CAT) is a more faithful marker for peroxisomes than PEX19, which we used previously to assess peroxisomal contamination²¹. When determining lysosomal contamination, either LAMP2 or CTSC can be used. We have, however, observed that the beads from both Control-MITO and HA-MITO IP samples bind to relatively low amounts of LAMP2, which probably represents binding of free LAMP2 that is not lysosomal in origin²¹. For this reason, we prefer CTSC, although the CTSC signal can be weak. Catalog numbers given are from Cell Signaling Technology and Santa Cruz Biotechnology.

Confocal microscopy and volumetric analysis of mitochondria ● **TIMING 3–4 d**

▲ **CRITICAL** This section is required for volumetric analysis, which is critical for absolute quantification of metabolites. However, for certain experiments, relative quantification of metabolites may be sufficient, in which case it is not necessary to include standard curves with every LC/MS run and perform confocal microscopy and volumetric analysis for each experimental condition, thus shortening the workflow considerably.

61 | Plate enough cells on a sterilized glass coverslip situated in a well of a 24-well plate so that the well is at least 70% confluent on the day of imaging. Although there can be variability in cell density across the coverslip, with some areas appearing 100% confluent and others appearing 70% confluent, this is generally acceptable. Different cell types may require different plating densities depending on their proliferation rates and cell sizes; for example, we plate 62,500 HeLa cells 2 d before processing them for imaging.

62 | To confirm proper mitochondrial localization of the Control-MITO and HA-MITO proteins, incubate the cells with medium containing 10 nM MDR for 1 h at 37 °C. If processing cells for volumetric analysis, it is not necessary to stain with MDR, but it is best to image HA-MITO cells that have experienced the same culture conditions (e.g., treatment with a drug) as those used for mitochondrial immunocapture to achieve experimental consistency.

63 | Aspirate off media, wash the stained cells with PBS and gentle swirling, and fix in 4% (wt/vol) PFA for 20 min.

! **CAUTION** PFA is toxic and should be handled in a chemical hood with appropriate disposal methods.

64 | Aspirate the overlying solution and wash with PBS and gentle swirling. Perform this step four times.

65 | Remove as much PBS as possible from the coverslips by gently dabbing them onto a Kimwipe. Place the coverslips onto a glass slide with 10 µl of anti-fade mounting medium containing DAPI.

66 | Cure in the dark at room temperature overnight.

67 | Place three small, equally spaced dots of nail polish around each coverslip the next day to help keep the coverslips in place.

■ **PAUSE POINT** Slides can be stored at 4 °C in the dark for at least several weeks.

PROTOCOL

68 | Image on a confocal microscope using the appropriate channels to confirm that EGFP properly colocalizes with MTDR and that the mitochondria appear morphologically normal (EGFP, MTDR, and DAPI channels) or to quantify the volume of the mitochondrial matrix (EGFP and DAPI channels).

? TROUBLESHOOTING

69 | To measure mitochondrial volume, generate z-stacks (step size = 0.25 μm) of at least 100 cells at 63 \times magnification for each experimental condition.

70 | Open all image files using Fiji and the Bio-Formats Importer plugin. For each image, view the stack as a 'Hyperstack'.

71 | Reset the brightness/contrast of each fluorescent channel.

72 | Export images as OME-TIFF files using the Bio-Formats Exporter plugin.

73 | Open the OME-TIFF files using the Imaris software and confirm that the x,y,z values for voxel size are correct with respect to the values listed under 'Properties' in the original Fiji file.

74 | Using Imaris, create 3D surfaces for the EGFP signal (surface area detail = 0.05 μm ; diameter of largest sphere that will fit into object = 0.25 μm) in each image. Note that the surface area detail and sphere diameter settings may vary depending on the mitochondrial morphology of the cell line being examined.

75 | Apply the built-in volumetric analysis to calculate the mitochondrial volume per cell for each image. Because images will sometimes have cells cut off at the borders, use the DAPI signal to identify cells and approximate the total number of cells included in a given image.

76 | For each set of images pertaining to one experimental condition, calculate the mean mitochondrial volume per cell.

77 | Note that the values obtained in Step 76 are not the mean matrix volumes, as the HA-MITO protein and its EGFP signal localize to the outer membrane of mitochondria. To determine the mean matrix volume per cell, multiply the values from Step 76 by 63.16%, which is the percentage of total mitochondrial volume occupied by the matrix in an orthodox configuration⁴². An alternative approach is to perform the volumetric analysis with a matrix-localized fluorescent protein. Although this requires introduction of another expression cassette into the cells, such a strategy is advantageous because it allows for direct visualization of changes in matrix volume without the assumption that the matrix is in the orthodox configuration. This can be useful, as prior *in vitro* work with isolated mitochondria has demonstrated that certain experimental perturbations can have effects on the volume of the mitochondrial matrix by pushing it out of the orthodox state⁴³.

▲ CRITICAL STEP The mean matrix volume per cell obtained here is the 'matrix volume' needed for Step 78.

Final calculations to determine the matrix concentration of a metabolite ● TIMING < 1 h

78 | Determine the final matrix concentration of a metabolite using the equations below, where units are given in parentheses:

$$\text{Total matrix volume} \left(\frac{\mu\text{l}}{\text{IP}} \right) = \text{Mitochondrial yield} \left(\frac{\text{cells}}{\text{IP}} \right) \times \text{Matrix volume} \left(\frac{\mu\text{l}}{\text{cell}} \right)$$

$$\text{Matrix metabolite concentration} (\mu\text{M}) = \frac{\text{Matrix metabolite abundance} \left(\frac{\text{pmol}}{\text{IP}} \right)}{\text{Total matrix volume} \left(\frac{\mu\text{l}}{\text{IP}} \right)}$$

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
49	Low or undetectable metabolite signal in HA-MITO IP	Contaminant is present in the reagents	Ensure that there are no large interfering peaks in the total ion chromatograms of KPBS, LC/MS PBS, and all other LC/MS reagents. If contaminants are present, then remake the solutions and strictly adhere to the instructions for preparing clean reagents for LC/MS work
		Insufficient mitochondrial material	Use the numbers of cells and beads recommended in the PROCEDURE. As discussed in Box 1 and Step 22 of the PROCEDURE, the required amount of homogenization can vary between cell lines and should also be optimized before attempting the workflow for a new cell line. Should these solutions not work, another option is to increase the sensitivity of the mass spectrometer for the metabolite of interest by performing a tSIM scan. We have found that tSIM scans notably improve detection of certain nucleotide species, such as CMP and GMP
		Extensive damage to mitochondria during isolation	As discussed in Box 1 and Step 22 of the PROCEDURE, the required amount of homogenization can vary between cell lines and should be optimized before attempting the workflow for a new cell line. In addition, one should strictly adhere to the instructions for washing the IP material to minimize mitochondrial damage. Across samples containing good-quality mitochondria, there should be a correlation between the amount of CS and the amount of total glutathione (GSH and GSSG) in the IP material
68	High background of MTDR	Concentration of MTDR used was too high	Perform a series of 2-fold dilutions of MTDR and determine which concentration gives the optimal balance between signal and background. There should not be too much nuclear and cytoplasmic staining when the concentration has been optimized

● TIMING

Steps 1–13, generation and validation of mammalian cells with epitope-tagged mitochondria: 11–15 d
 Steps 14–37, isolation and processing of mitochondria: 1–2 h per sample (including Steps 23–32, posthomogenization workflow until metabolite extraction: ~12 min)
 Steps 38–50, absolute quantification of metabolites by LC/MS: 1–2 d
 Steps 51–60, immunoblot analysis of the IP material: 2–3 d
 Steps 61–77, confocal microscopy and volumetric analysis of mitochondria: 3–4 d
 Step 78, final calculations to determine the matrix concentration of a metabolite: <1 h
Box 1, optimization of cellular homogenization: 1.5–10 h, depending on the number of homogenization conditions examined

ANTICIPATED RESULTS

The protocol described above details the steps needed to rapidly immunopurify mitochondria and quantify matrix concentrations of mitochondrial metabolites. The relevant controls have been provided to assess the quality of the data. Although there may be variability between different cell lines, there should be substantial enrichment of mitochondria relative to other subcellular compartments when examining the IP material by immunoblotting (**Fig. 2b**).

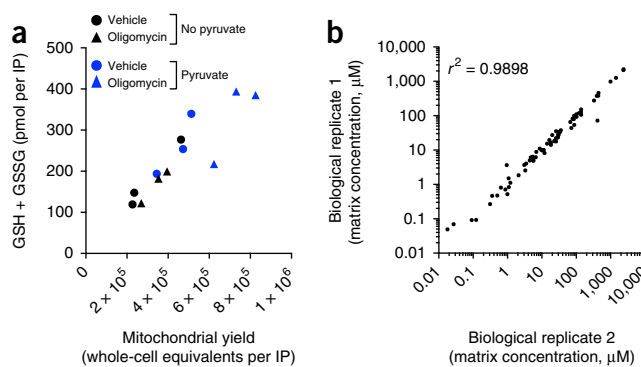


Figure 3 | Quantitative benchmarks for assessing reliability of data. (a) For cells cultured under a variety of conditions, the total abundance of GSH and GSSG in the HA-MITO IP increases proportionately with the amount of isolated mitochondria. The amount of mitochondria isolated in each IP is represented as whole-cell equivalents and is assessed using immunoblot analysis of citrate synthase, a protein that localizes predominantly to the mitochondrial matrix²⁴. HeLa cells were cultured in complete DMEM or DMEM without pyruvate and treated for 2 h with 100% DMSO (Vehicle) or 5 μM oligomycin as indicated. Each point is a biological replicate for its respective culture condition. (b) Measurements of matrix metabolites are highly consistent between experiments. HeLa cell matrix concentrations from two biological replicates were compared, and a Pearson's correlation coefficient was calculated. Each dot represents a different mitochondrial metabolite. HeLa cells were cultured in complete DMEM. For all experiments in this figure, HeLa cells were authenticated by the Duke University DNA Analysis Facility, and tested for mycoplasma contamination. This figure was generated using the resource data made available through our prior work²¹.

Typically, if using 30 million HeLa cells and 200 μ l of beads, the expected yield is ~500,000 whole-cell equivalents in the entire HA-MITO IP material. With regard to metabolites, this yield corresponds to ~118 pmol of total NAD and NADH, and ~296 pmol of total GSH and GSSG. Depending on the metabolite of interest, lower amounts of cells and/or beads may be used. With regard to GSH and GSSG, we have found that the combined abundance of these glutathione species provides a useful proxy for mitochondrial yield. In particular, for isolated mitochondria that retain both their matrix protein and matrix metabolite contents, the combined amount of GSH and GSSG should increase with the mitochondrial yield, as determined by immunoblot analysis, in a roughly proportional manner. This phenomenon is quite robust and occurs in different culture conditions and across different states of respiratory chain function (**Fig. 3a**). As such, assuming that the experimental conditions being used are not changing total glutathione levels within the mitochondrial matrix, we feel that the concordance between mitochondrial yield and total GSH and GSSG abundance in the IP material provides a reasonable metric for assessing the relative quality of mitochondria across multiple isolations. In general, the entire workflow for quantifying matrix metabolite concentrations is quite robust when optimized, with substantial reproducibility between biological replicates (**Fig. 3b**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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