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

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## Abstract

Mitochondria carry out numerous metabolic reactions that are critical for cellular homeostasis. Here we present a protocol for interrogating mitochondrial metabolites and measuring their matrix concentrations. Our workflow utilizes high-affinity magnetic immunocapture to rapidly purify HA-tagged mitochondria from homogenized mammalian cells in ~12 minutes. 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, while 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 purity of isolated mitochondria compared to 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 of the necessary reagents have been prepared, quantifying the matrix concentrations of mitochondrial metabolites can be accomplished within a week.

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**AUTHOR CONTRIBUTIONS:**

W.W.C. and D.M.S initiated the project and designed the research. E.F. played an invaluable role in establishing the LC/MS platform and designing the metabolomics methodology. W.W.C. and D.M.S. wrote and edited the manuscript.

**COMPETING FINANCIAL INTERESTS:**

Walter W. Chen is a consultant for VL39, a company developing novel therapeutic modalities for treating mitochondrial pathologies.

## INTRODUCTION

Mitochondria are membrane-bound organelles that house numerous chemical reactions essential for 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 cells[10]. 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 (Figure 1). The method utilizes high-affinity magnetic immunopurification (IP) of HA-tagged mitochondria to achieve rapid isolation of mitochondria within 12 minutes following cellular homogenization. Mitochondrial metabolites are interrogated using liquid chromatography and mass spectrometry (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 needs to 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/MS[19]. 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 metabolite[21].

To address these challenges, we developed a workflow utilizing high-affinity magnetic immunocapture that is both LC/MS-compatible and capable of rapidly and specifically isolating mitochondria for metabolite profiling[21]. Because small molecules interfere with LC/MS analysis, we designed a mitochondrial isolation buffer composed of only KCl and $KH_2PO_4$, which we named “KPBS.” Consistent with previous literature using KCl-based

buffers[22], we found that KPBS not only maintained mitochondrial integrity but also improved the quality of LC/MS data dramatically[21].

To achieve rapid and specific isolation of mitochondria, we employed a strategy whereby an outer mitochondrial membrane 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[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 for mitochondrial isolation. While 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 likely too small for mitochondria (~500 nm diameter) to enter. As such, the majority of the immunocapture is likely 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 μm to 1 μm dramatically increases the amount of mitochondria that are isolated[21]. Collectively, these improvements allow our LC/MS-compatible methodology to rapidly purify mitochondria within 12 minutes of homogenizing 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 (Figure 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, while 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 matrix[24], 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 metabolites[21]. 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 metabolome[21].

## 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 dramatically 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 where viral transduction is undesirable, these constructs can also be transfected into cells prior to 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 employed 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 play important roles in mammalian physiology, such as the lysosome and 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 give 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, non-aqueous 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 utilizing 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 non-specifically 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 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 compared to the whole-cell material. Additional controls for assessing the purity of the IP also include measuring 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. Each plate of cells is 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 likely 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 post-doctoral 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—**While this workflow is generally robust and quite reproducible, there are some technical points to consider. Thermo Fisher Scientific offers both 1 μm anti-Myc and 1 μm 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 while the HA-MITO IP sample has substantially reduced amounts of extra-mitochondrial material compared to whole cells, we have found that our workflow removes endoplasmic reticulum (ER) less efficiently than lysosomes and peroxisomes. This is likely 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 likely varies across cell lines depending on the strength of mitochondrial-ER contacts. Thus, while 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. While we have successfully been able to study matrix metabolites using less than 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 where detection is poor due to decreased starting material, we have had success with targeted selected ion monitoring (tSIM) scans, which can improve metabolite signals considerably on the LC/MS; metabolites that have notably benefited from tSIM scans include certain nucleotide species, such as CMP and GMP.

In our prior work, we show that lengthening the post-homogenization time preceding metabolite extraction from 12 minutes to 16 minutes does not lead to a substantial distortion of the matrix metabolite profile[21], 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 minutes, 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: 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% (w/v) 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 due 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 non-specifically 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)
- Dimethyl sulfoxide (DMSO) (EMD Millipore, cat. no. MX1458-6)
- DMEM powder (Sigma-Aldrich, cat. no. D7777)
- Ethanol, 190 proof (Pharmco-Aaper, cat. no. AAP-111000190CSGL) ! CAUTION: Highly flammable liquid and vapor.
- Ethylenediaminetetraacetic acid (EDTA) solution, 500 mM, pH 8.0 (Thermo Fisher Scientific cat. no. AM9260G) !CAUTION: EDTA is toxic so avoid exposure.
- BenchMark fetal bovine serum – 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. CRITICAL: The workflow may require minor modifications if using different cell types.

  !CAUTION: Ensure cells remain free of mycoplasma and regularly check the authenticity of your cells in culture.

  HEK293T cells (GenHunter Corporation, cat. no. Q401) !CAUTION: Ensure cells 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: 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 Number MSK-A2-1.2)
- Methanol, Optima LC/MS grade (Thermo Fisher Scientific, cat. no. A456-4) ! CAUTION: 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)
- Non-fat dry milk (LabScientific, cat. no. M-0842)

- Novex WedgeWell 12% Tris-glycine gels, 15 well (Thermo Fisher Scientific, cat. no. XP00125BOX)
- Paraformaldehyde (PFA), 16% (w/v) solution (Electron Microscopy Sciences, cat. no. 15710) !CAUTION: 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% (w/v) solution (Sigma-Aldrich, cat. no. P0290)
- Plasmids carrying the *gag*, *pol* genes (Addgene plasmid #8449)[35] and *vsv-g* gene (Addgene plasmid #8454)[35]. Plasmids carrying the *3XMyc-EGFP-OMP25* gene (Control-MITO construct, Addgene plasmid #83355)[21] and *3XHA-EGFP-OMP25* gene (HA-MITO construct, Addgene plasmid #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 ($KH_2PO_4$) (Sigma-Aldrich, cat. no. P5655)
- Potassium hydroxide (KOH), 45% (w/w) solution (J.T. Baker, prod. no. 3143-01) !CAUTION: Corrosive and toxic.
- Protease inhibitor cocktail tablets (Roche, cat. no. 05 892 791 001)
- PVDF membranes, 0.45 μm pores (Thermo Fisher Scientific, cat. no. IPVH00010)
- Sodium bicarbonate (US Biological, cat. no. S4000)
- Sodium chloride (NaCl) (Sigma-Aldrich, cat. no. S9888)
- Sodium dodecyl sulfate (SDS) pellets (Sigma-Aldrich, cat. no. 75746)
- Sodium hydroxide (NaOH), 50% (w/w) solution (J.T. Baker, prod. no. 3727-01) ! CAUTION: Corrosive and toxic.
- Sodium hydroxide pellets (NaOH pellets) (AmericanBio, cat. no. AB01916-01000) !CAUTION: Corrosive and toxic.
- Sodium phosphate dibasic ($Na_2HPO_4$) (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 HardSet 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 (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 2.1 × 150 mm, 5 μm particle size, column – analytical software is XCalibur Quanbrowser version 2.2 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 (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 Eppendorf 5424 microcentrifuge with FA-45-24-11 rotor)
- Milli-Q water system (e.g., Milli-Q water purification system with Biopak Polisher)
- Minifuge, Galaxy Mini (VWR, model number 93000-196)
- Orbiter (e.g., The Belly Dancer Shaker, IBI Scientific model number BDRAA115S)
- Owl VEP-2 mini tank electroblotting system (Thermo Fisher Scientific, cat. no. VEP-2)
- pH meter (e.g., Beckman Coulter pHI 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 Allegra X-12R with 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 DMEM powder, 37 g sodium bicarbonate, and 3 g L-glutamine in 10 L Milli-Q water. Adjust the pH to 7 using HCl, filter into sterile, autoclaved glass bottles using a 0.22 μm filter-top, and store 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 (P/S)—**Add 100 mL of stock solution to 100 mL of Milli-Q water, aliquot, and store at −20°C. These aliquots are stable for at least 1 month.

**Complete DMEM—**Complete DMEM is base DMEM supplemented with 10% (v/v) IFS, 1% (v/v) P/S. Add 50 mL IFS, 5 mL P/S to 450 mL base DMEM. Filter into a sterile, autoclaved glass bottle using a 0.22 μm filter-top and store 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 $Na_2HPO_4$, 2.7 mM KCl, 1.47 mM $KH_2PO_4$, pH 7.1, in Milli-Q water. Dissolve 8 g of NaCl, 1.14 g of $Na_2HPO_4$, 200 mg of KCl, and 200 mg of $KH_2PO_4$ in 800 mL of Milli-Q water. Adjust pH to 7.1 with HCl and bring volume up to 1 L 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% (w/v) trypsin, 2.4 mM EDTA, 0.0005% (w/v) 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% (w/v) solution). Bring volume up to 1 L with additional sterile PBS and adjust pH to 7 with HCl. Filter with a 0.22 μm filter-top, aliquot, and store 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, 0.1% (w/v) ammonium hydroxide. Dissolve 0.481 g of ammonium carbonate in 240 mL LC/MS grade water. Add 892 μL of ammonium hydroxide (28% (w/v) 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% (v/v) LC/MS grade methanol, 20% (v/v) 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 μL of the metabolomics mix containing 17 isotopically-labeled amino acids. Aliquot solution into Wheaton glass bottles and store at −20°C. This solution is stable for at least several months.

**KPBS—**KPBS is 136 mM KCl, 10 mM $KH_2PO_4$, 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 KCl and 0.68 g $KH_2PO_4$ in 450 mL LC/MS grade water. pH to 7.25 using KOH and bring the volume up to 500 mL with LC/MS grade water. Filter through a 0.22 μm filter-top bottle and store 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 prior to mitochondrial immunopurification and is 137 mM NaCl, 8 mM $Na_2HPO_4$, 2.7 mM KCl, 1.47 mM $KH_2PO_4$, pH 7.1, in LC/MS grade water. Dissolve 8 g of NaCl, 1.14 g of $Na_2HPO_4$, 200 mg of KCl, and 200 mg of $KH_2PO_4$ in 800 mL of LC/MS grade water. Adjust pH to 7.1 and bring volume up to 1 L with additional LC/MS grade water. Filter through a 0.22 μm filter-top bottle and store 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 water. Adjust the pH to 7.4 using HCl and bring volume up to 100 mL. Store at 4°C. The solution is stable for at least 1 year.

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

**20% (v/v) Triton X-100—**Add 20 mL of Triton X-100 to 80 mL water. Store 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% (v/v) Triton X-100, and protease inhibitor. Add 5 mL of 1 M Tris-HCl, pH 7.4, 3 mL of 5 M NaCl, 200 μL of 500 mM EDTA, pH 8.0, 5 mL of 20% (v/v) Triton X-100, and bring up the volume to 100 mL with water. Store 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. Aliquot and store resulting lysis buffer at −20°C. These aliquots are stable for at least 1 month but avoid freeze-thawing.

**5X Laemmli sample buffer (5X LB)—**This buffer is 237 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, and 0.056% (w/v) bromophenol blue. Dissolve 1.68 g Tris-HCl powder, 4.5 g SDS in 22.5 mL water. Add 11.25 mL 2-mercaptoethanol, 11.25 mL glycerol, pH to 6.8 with NaOH first, and then add 0.025 g of bromophenol blue. Pass solution through a 0.45 μm filter, aliquot, and store at −20°C. These aliquots are stable for at least several months. !CAUTION: NaOH is corrosive and toxic so handle it with care.

**10X TBST—**This buffer is 1.37 M NaCl, 200 mM Tris-HCl, pH 7.6, and 1% (v/v) Tween-20. Dissolve 160 g NaCl, 50.1 g Tris-HCl powder, and 9.91 g Tris in 1.7 L water. Add 20 mL Tween-20, pH to 7.6, bring up to 2 L with water, and store at room temperature. The 10X buffer is stable for at least several months. Dilute to 1X with water before use.

**10X SDS running buffer—**This buffer is 248 mM Tris, 1.92 M glycine, and 1% (w/v) SDS. Dissolve 30 g of Tris, 144 g of glycine, and 10 g of SDS in 1 L of water. Store at room

temperature. The 10X buffer is stable for at least several months. Dilute to 1X with water before use.

**Transfer buffer—**This buffer is 10 mM CAPS, 10% (v/v) ethanol. Add 200 mL of 190 proof ethanol to 1.8 L 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% (w/v) milk—**Dissolve 20 g of non-fat dry milk in 400 mL of 1X TBST. Store at 4°C. The milk is usable only for 2 weeks.

**4% (w/v) PFA—**Add 9 mL of 16% (w/v) PFA to 27 mL of PBS. Aliquot and store at −20°C. The solution is stable for at least several months but avoid multiple freeze-thaws. ! 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 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 trypsin, so it is important to remove residual trypsin from the cell suspension by centrifugation and resuspension in fresh media when plating.

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

| Component | Amount per 6 cm dish |
|---|---|
| *gag-pol* construct | 1 μg |
| *vsv-g* construct | 0.1 μg |
| Control-MITO or HA-MITO constructs | 1 μg |
| X-tremeGENE 9 | 6 μL |

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

4. Add the mixtures from step 2 drop-wise around the plate, gently swirl to mix, and incubate cells for 18–24 h.

5. Replace media of each plate with 4 mL of fresh complete DMEM and wait 24 h.

**6** Collect the media while trying to minimize contamination with HEK293T cells and then pass through a 0.45 μm 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 cycles of freezing and thawing, we recommend making multiple aliquots that do not undergo more than two freeze-thaws.

**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 (e.g., HeLa cells) in 3 mL of media 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 media 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 – 50 μL.

**9** Centrifuge the plate at ~1,100*g* 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 media 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 wildtype cells in the FACS analysis for establishing gates. Note that while 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 similar amounts of mitochondrial capture to higher-expressing systems while substantially reducing contamination with organelles such as the peroxisome (Figure 2, Supplementary Figure 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 when

compared to whole-cell lysates. Cells should also be visually inspected to ensure that there is proper colocalization of EGFP with the mitochondrial dye, MTDR, 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 usage, freeze multiple aliquots of Control-MITO and HA-MITO cells in cryogenic vials using fresh-made, chilled growth media containing 10% (v/v) 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.

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

### Mitochondrial isolation, and metabolite and protein extraction. TIMING: For one sample, steps 14–37 take 1–2 h. Steps 23–32 (post-homogenization workflow until metabolite extraction) take ~12 min

CRITICAL: Perform steps 14–15 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 homogenous suspension. Aliquot 200 μL of anti-HA beads to a separate 1.5 mL tube for each IP using Control-MITO or HA-MITO cells.

**15** Collect 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 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 homogenous. 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 bead suspension on ice when done.

**16** Move to the cold room so 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 media and aspirate any residual liquid. Note that while this protocol has been designed for adherent cells, it can in theory be adapted for suspension cells as well.

**18** Pour 30 mL of pre-measured, ice-cold LC/MS PBS onto cells and wash cells by swirling the liquid around the plate and then rocking 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 cells into KPBS with the scraper's non-angled face oriented away from the cells. To avoid unnecessary stress on the cells, harvest 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 get a homogenous 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 approximately 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. While 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 prior to attempting mitochondrial isolation (Box 1, Supplementary Figure 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 previously[40].

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

**24** Collect 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 μL 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 homogenous.

**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 beads on the magnet and thoroughly aspirate the overlying solution as described in step 15. Collect 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 beads. To wash beads, gently pipette up and down five times. The solution should be homogenous 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 performing the IP purely for immunoblot analysis, proceed directly to step 34 with the 1 mL suspension of beads.

**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 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 homogenous and incubate on ice for 5 min. CRITICAL STEP: To extract lipids and more non-polar 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 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 finished, 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 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 5X LB, vortex to mix, incubate at 100°C for 5 min, pulse spin to collect material on the lid, and store at −20°C. PAUSE POINT: Samples can be stored at −20°C for up to a year.

**Box 1**

**Optimizing the homogenization of cells. TIMING: 1.5–10h (depending on the number of homogenization conditions examined)**

Determining the minimum amount of homogenization necessary for sufficient cell rupture is critical for 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 μL 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 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 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 homogenous single cell suspension.

**4.** Gently mix 15 μL of the cell suspension with 985 μL of KPBS and leave this 1 mL solution of diluted cells on ice for later use. This sample contains non-homogenized cells.

5. Transfer the remaining cell suspension to the homogenizer and apply increasing amounts of homogenization, making sure to gently mix 15 μL of the homogenate with 985 μL 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 non-rotating 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 the well of a 6 well plate, gently rock the plate back and forth to evenly distribute the contents, and visualize cells by microscopy. Based on our experience and the existing literature[30], 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 plugin, Cell Counter, and calculate the ratio of total cells to total free nuclei. We typically examine enough images to accrue 500–1000 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 every free nuclei and enables successful profiling of mitochondrial metabolites. As such, the minimal amount of homogenization that can give 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 sub-optimal.

### 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 can be modified for studying lipids and other non-polar 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 every metabolite to be analyzed using relative quantification or absolute quantification, first run authentic standards on the LC/MS 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 metabolism[21].

**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 storing 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 nM, 10 nM, 100 nM, 1 μM, 10 μM, 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 prior to extraction of an IP.

**40** Using a Dionex UltiMate 3000 UPLC system, inject 5 μL of each sample onto a ZIC-pHILIC 2.1 × 150 mm (5 μm particle size) column. No dilutions or modifications are made to the sample after extraction from the beads and prior to injection on the LC/MS. For every run, also inject 5 μL of a sample containing 5 μL of KPBS mixed with 50 μL of extraction solution; this sample will be used to assess the purity of the KPBS and should generally have low to undetectable metabolite signal compared to 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 ±3.0 kV, the heated capillary set to 275°C, and the HESI probe set to

350°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 70–1000 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 targeted selected ion monitoring (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 work[21].

**45** Identify and quantify metabolites using XCalibur QuanBrowser version 2.2 or higher. Set the mass accuracy window to 10 ppm 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 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, every 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 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.

**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:

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

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

### 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 appropriate amount of 5X 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 comprised 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 cells[24]. In addition, its solubility[41] and residence within the matrix[24] make CS a good proxy for mitochondria with intact inner membranes.

**52** Load 15 μL of IP 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 membrane in 5% (w/v) milk for 45 min at room temperature.

**55** Incubate membrane for 24–48 h with the desired primary antibodies at 4°C. For quantifying whole-cell equivalents in the IP material, only CS needs to 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 membrane three times with TBST, 5 min each.

**57** Incubate membrane with the appropriate secondary antibody (diluted 1/5000 in 5% (w/v) 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.

### 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. While 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** For confirming proper mitochondrial localization of the Control-MITO and HA-MITO proteins, incubate cells with media containing 10 nM MTDR for 1 h at 37°C. If processing cells for volumetric analysis, it is not necessary to stain with MTDR, 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 stained cells with PBS and gentle swirling, and fix in 4% (w/v) PFA for 20 min. !CAUTION: PFA is toxic and should be handled in a chemical hood with appropriate disposal methods.

**64** Aspirate overlying solution and wash with PBS and gently swirling. Perform this step four times.

**65** Remove as much PBS as possible from the coverslips by gently dabbing them onto a kimwipe. Place coverslips onto a glass slide with 10 μL of anti-fade mounting media 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.

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

**69** For measuring mitochondrial volume, generate z-stacks (step size = 0.25 μm) of at least 100 cells at 63X magnification for every experimental condition.

**70** Open all image files using Fiji and the plugin, Bio-Formats Importer. 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 plugin, Bio-Formats exporter.

**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** Create three-dimensional 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 using Imaris. 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 configuration[42]. An alternative approach is to perform the volumetric analysis with a matrix-localized fluorescent protein. Although this requires introduction of another expression cassette into 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 state[43].

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:

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

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

## TIMING

Steps 1–13, generation and validation of mammalian cells with epitope-tagged mitochondria: 11–15 d.

Steps 14–37, mitochondrial isolation, and metabolite and protein extraction: 1–2 h.

(including Steps 23–32, post-homogenization 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, optimizing the homogenization of cells: 1.5–10h (depending on the number of homogenization conditions examined).

### TROUBLESHOOTING

Troubleshooting guidance is in Table 2.

## 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 (Figure 2b). Typically, if using 30 million HeLa cells and 200 μL of beads, the expected yield is ~500,000 whole-cell equivalents in the entire HA-MITO IP material. Metabolite-wise, 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 regards 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 proportionate manner. This phenomenon is quite robust and occurs in different culture conditions and across different states of respiratory chain function (Figure 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 (Figure 3b).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## EDITORIAL SUMMARY

High-affinity magnetic immunocapture is used to rapidly purify HA-tagged mitochondria from cells for metabolite profiling. Matrix concentrations of mitochondrial metabolites are determined through LC/MS, immunoblotting, confocal microscopy, and volumetric analysis.

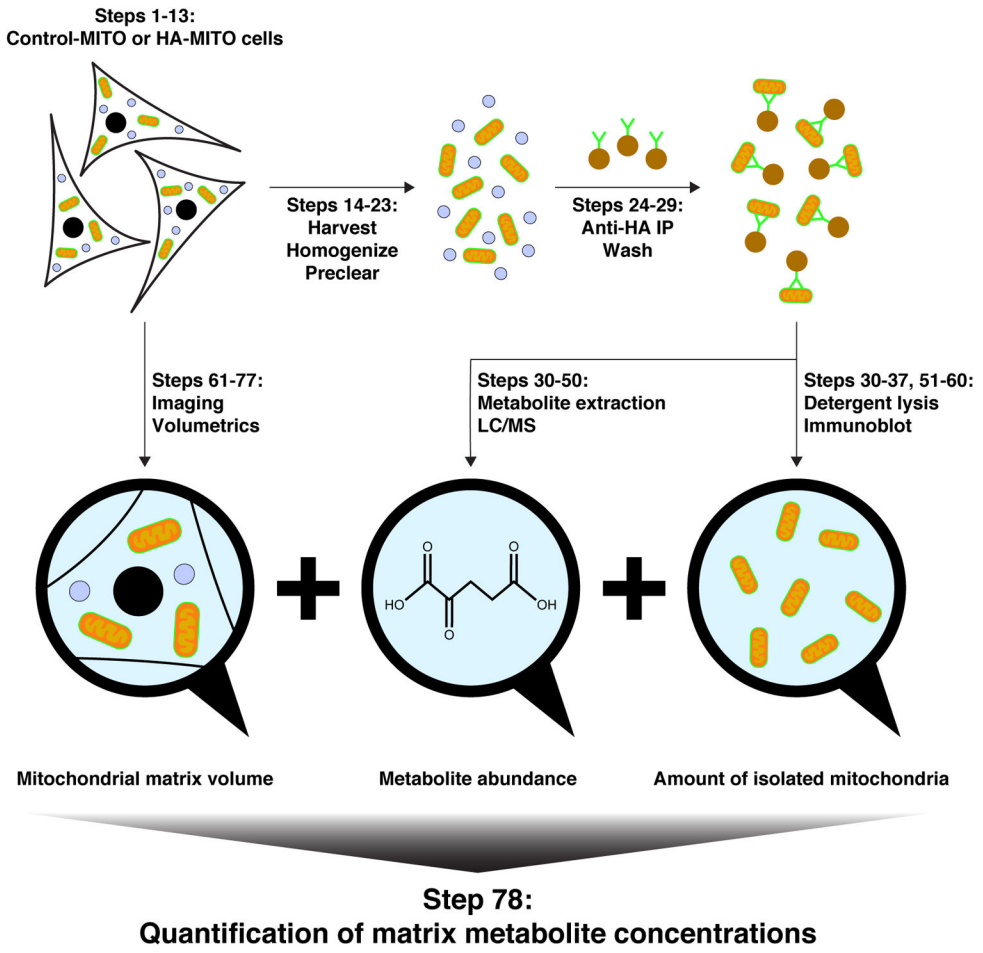


**Figure 1. Workflow for quantifying matrix concentrations of mitochondrial metabolites**

Step numbers in the figure correspond to those in the PROCEDURE of the manuscript. 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 immunopurification (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 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).

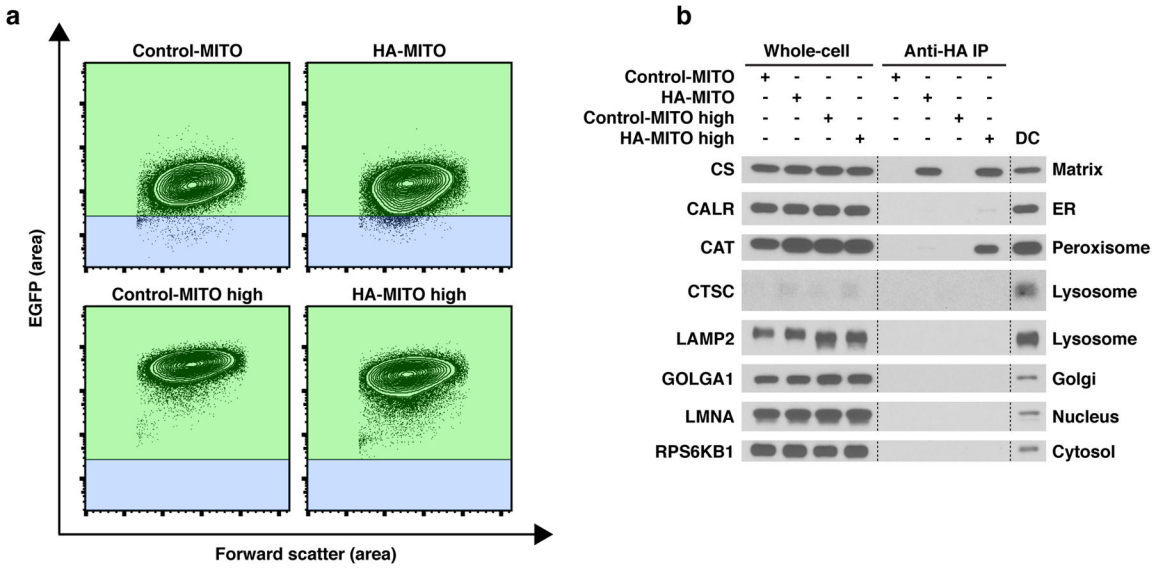


**Figure 2. The degree of HA-MITO construct expression affects the purity of isolated mitochondria**

**(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 was 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 previously[21]. 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. Matrix, mitochondrial matrix; ER, endoplasmic reticulum; Golgi, Golgi complex. 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.

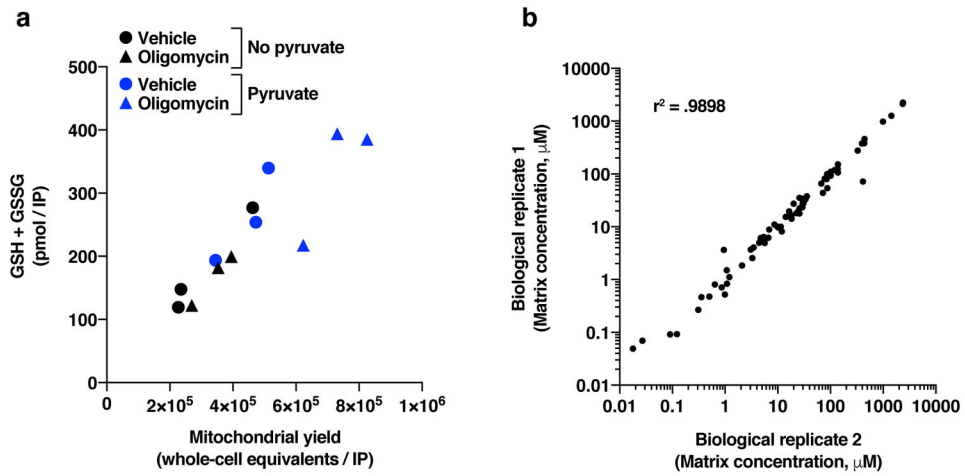


**Figure 3. Quantitative benchmarks for assessing reliability of data**

**(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 matrix[24]. 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 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 work[21].

## 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) | 1/1000 |
| RPS6KB1 | 50–80 | Cytosol | CST anti-RPS6KB1 antibody (cat. no. 2708) | 1/1000 |
| CALR | 60 | Endoplasmic reticulum | CST anti-CALR antibody (cat. no. 12238) | 1/1000 |
| GOLGA1 | 90 | Golgi complex | CST anti-GOLGA1 antibody (cat. no. 13192) | 1/1000 |
| LAMP2 | 100–140 | Lysosome | SCBT anti-LAMP2 antibody (cat. no. sc-18822) | 1/1000 |
| 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) | 1/1000 |
| CAT | 60 | Peroxisome | CST anti-CAT antibody (cat. no. 12980) | 1/1000 |

CRITICAL: We have found that catalase (CAT) is a more faithful marker for peroxisomes than PEX19, which we used previously to assess peroxisomal contamination[21].

CRITICAL: 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 low levels of LAMP2 that is likely free LAMP2 and not lysosomal in origin[21]. For this reason, we prefer CTSC, although the CTSC signal can be weak.

**Table 2**

Troubleshooting

| Step | Problem | Possible reason | Possible solution |
|---|---|---|---|
| 49 | Low or undetectable metabolite signal in HA-MITO IP | Contaminant in 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 solutions and strictly adhere to the instructions for preparing clean reagents for LC/MS work. |
| | | Insufficient mitochondrial material | Use the number 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. |