Chapter 15

Human Mitoribosome Profiling: A Re-engineered Approach Tailored to Study Mitochondrial Translation

Iliana Soto, Mary Couvillion, and L. Stirling Churchman

Abstract

To understand the human mitochondrial translation process, tools are required to dissect this system at a global scale. The mechanisms and regulation of translation in mitochondria are different from those in the cytosol, and mitochondrial ribosomes have distinct biochemical properties. In this chapter, we describe in detail the modifications we have made to the ribosome profiling approach to adapt it to the unique characteristics of the human mitochondrial ribosome. This approach maximizes the fraction of mitochondrial ribosomes recovered, providing a snapshot of the mitochondrial translation landscape with minimal bias. We also describe the use of mouse lysate as an internal spike-in control for normalization, allowing quantification of global changes in translation across samples. Finally, we outline the bioinformatic pipelines to process the raw reads and identify mitoribosome A sites in the absence of untranslated regions flanking open reading frames. This method offers a subcodon-resolution time-sensitive global approach to explore the mitochondrial translation process in human cells.

Key words Human mitochondrial translation, Mitochondrial ribosome profiling

1 Introduction

The mechanisms and regulation of mitochondrial translation have been challenging to study, and gaps exist in our knowledge despite the plethora of pathologies caused by mutations in the mitochondrial translation machinery [1, 2]. The mitochondrial translation response to environmental and developmental changes is currently unexplored because tools have not been developed for the quantitative, time-resolved studies of mitochondrial translation in vivo. In recent years, mitochondrial translation has been studied using ribosome profiling conditions that had previously been tailored to study translation in the cytosol [3]. Mitochondrial ribosomes (mitoribosomes) are entirely distinct from cytoplasmic ribosomes (cytoribosomes) and have different biochemical properties. They have a higher protein to RNA ratio, as a result of a contraction of ribosomal RNA and the acquisition of several additional proteins, which

Antoni Barrientos and Flavia Fontanesi (eds.), *The Mitoribosome: Methods and Protocols*,
Methods in Molecular Biology, vol. 2661, https://doi.org/10.1007/978-1-0716-3171-3_15,

facilitate the particular requirements of protein synthesis in mitochondria [4], requiring the re-engineering of existing ribosome profiling methods for the study of mitochondrial translation. The improved protocol we present in this chapter offers a powerful tool to better study mitochondrial gene expression and to gain insight into the translation process itself.

Ribosome profiling has previously been applied to study mitochondrial translation in human cells [5–7]. Despite high-sequencing depth (50–150 million reads per library), these approaches display incomplete coverage over mitochondrial open reading frames, potentially due to capture inefficiencies [8]. Here we present a method optimized at each step (*see* Fig. 1) to enrich mitochondrial ribosome-protected fragments (mitoRPFs) over the more abundant cytosolic ribosome-protected fragments (cytoRPFs), achieving 97–100% coverage over all mitochondrial codons in multiple cell types from a sequence depth of 50–150 million reads. First, because the subunits of the mitoribosome tend to dissociate more readily than those of the cytoribosome and must be released from the mitochondrial membrane, we use relatively low salt and high magnesium conditions along with a specialized detergent (lauryl maltoside) to preserve the integrity of mitochondrial polysomes while fully extracting them from the mitochondrial

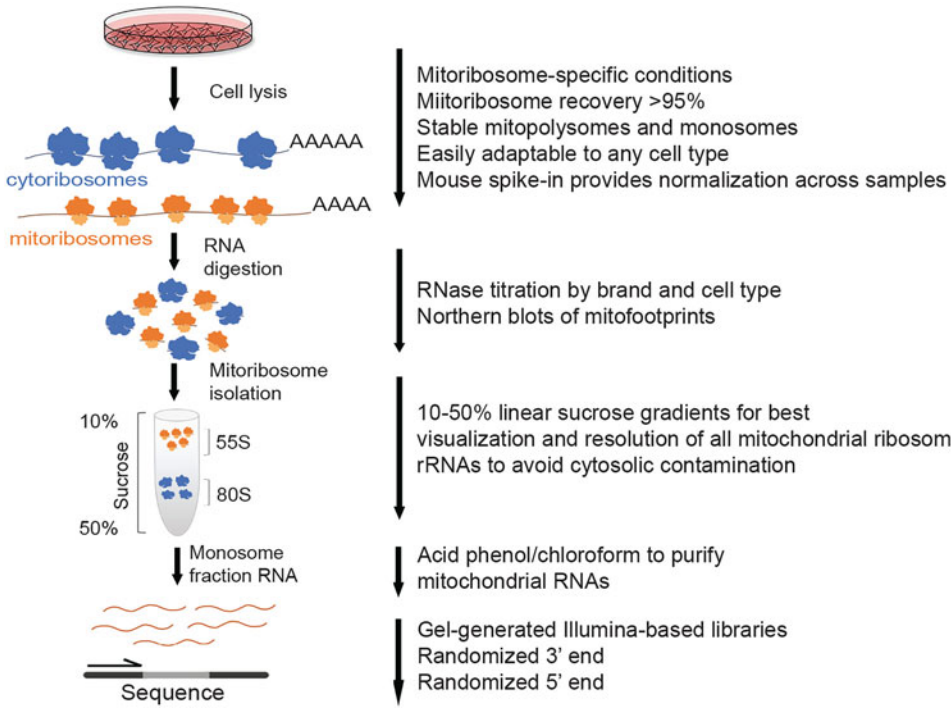

Fig. 1 Schematic of the human mitoribosome profiling approach. We describe the modifications to each step of the approach, from sample collection to building and sequencing libraries

inner membrane [9]. Second, we titrated RNase I and found that higher concentrations should be used compared with cytosolic ribosome profiling, likely because of the enhanced membrane solubilization and absence of lysate clarification step. Additionally, the mitoribosome contains less structural RNA than the cytoribosome making it more resistant to degradation by RNase treatment. Third, the gradient used for fractionation has the resolution to separate mitochondrial ribosomes (mitomonosomes, 55S) from cytosolic ribosomes (cytomonosomes, 80S). Immunoblotting is required to track these separate fractions as mitoribosomes are not abundant enough to produce a peak of absorbance at 260 nm. Fourth, we include a unique molecular identifier (UMI) in order to detect and remove duplicate reads that result from PCR amplification, producing an accurate quantitative measure of mitoribosome occupancy across the mitochondrial transcriptome.

Developmental, environmental, and genetic perturbations can cause drastic changes in mitochondrial gene expression, and with only 13 protein-coding genes, these changes are often global. We describe here the use of mouse lysate to serve as a spike-in control so that these global changes can be observed.

Complementary to the improvement of sample generation by ribosome profiling, we also designed novel bioinformatic tools. Although many computational packages are available for the analysis of ribosome profiling data, the peculiarities of mammalian mitochondrial transcripts require special attention. In particular, the absence of untranslated regions in most mRNAs makes A site determination challenging. Here, we provide the main steps for data analysis including the use of mouse mitoRPFs as spike-in controls, with special focus on determining the offset for A site transformation. The optimization of these steps from sample collection to data analysis provides a robust method to monitor translation in human mitochondria and limit bias.

2 Materials

All solutions should be prepared with nuclease-free water and only nuclease-free tubes should be used.

2.1 Cell Growth

1. Complete Dulbecco's Modified Eagle Medium (DMEM), high glucose formulation, containing sodium pyruvate.
2. Fetal bovine serum (FBS).
3. Cosmic calf serum.
4. HeLa S3 and NIH3T3 mouse fibroblasts cell lines.

2.2 Cell Lysis and Sample Collection

1. 1× PBS pH 7.4.
2. 5% Lauryl maltoside stock solution in nuclease-free water.
3. 5 M NH_4Cl stock solution in nuclease-free water.
4. 1 M $MgCl_2$ stock solution in nuclease-free water.
5. 0.1 M DTT stock solution in nuclease-free water.
6. 1 M Tris–HCl pH 7.5 stock solution in nuclease-free water.
7. 50× EDTA-free protease inhibitor cocktail stock solution in nuclease-free water.
8. *Lysis buffer*: 0.25% Lauryl maltoside, 50 mM NH_4Cl, 20 mM $MgCl_2$, 0.5 mM DTT, 10 mM Tris, pH 7.5, 1× EDTA-free protease inhibitor cocktail (*see* **Note 1**).
9. Loose-fit glass/glass 5 mL homogenizer.

2.3 RNase Digestion

1. RNase I.
2. SuperaseIn.
3. NanoDrop instrument.

2.4 Mitochondrial Ribosome Isolation

2.4.1 Sucrose Gradient Sedimentation

1. Ultracentrifuge tubes.
2. Ultracentrifuge tubes, rubber stoppers, and 10 cm cannula (Biocomp).
3. 60% filter-vacuumed sucrose stock in nuclease-free water.
4. 5% Lauryl maltoside stock solution in nuclease-free water.
5. 5 M NH_4Cl stock solution in nuclease-free water.
6. 1 M $MgCl_2$ stock solution in nuclease-free water.
7. 0.5 M EDTA, pH:8 stock solution in nuclease-free water.
8. 0.1 M DTT stock solution in nuclease-free water.
9. 1 M Tris pH 7.5 stock solution in nuclease-free water.
10. 50× EDTA-free protease inhibitor cocktail stock solution in nuclease-free water.
11. *50% sucrose mix*: 50% sucrose, 0.1% lauryl maltoside, 50 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM DTT, 10 mM Tris, pH 7.5, 1× EDTA-free protease inhibitor cocktail (*see* **Note 1**).
12. *10% sucrose mix*: 10% sucrose, 0.1% lauryl maltoside, 50 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM DTT, 10 mM Tris, pH 7.5, 1× EDTA-free protease inhibitor cocktail (*see* **Note 1**).
13. Beckman Ultracentrifuge and SW 41 Ti Swinging Bucket Rotor.
14. Biocomp sucrose gradient master and fractionator instrument.

2.4.2 Mitochondrial Ribosome Visualization in Sucrose Gradients

1. *5× sample loading buffer for Western blots*: 4% SDS, 250 mM Tris pH 6.8, 250 mM DTT, 30% glycerol, and 0.6 mg/mL bromophenol blue (Aliquot and store at −20 °C).
2. 15% Bis-Tris polyacrylamide precast gels.
3. 20% MOPS western running buffer (1 M MOPS, 1 M Tris, 2% SDS, 20 mM EDTA).
4. Pre-stained protein ladder.
5. MRPL12 (Proteintech, 14795-1-AP) and MRPS18-B (Proteintech, 16139-1-AP) antibodies.
6. Skim milk powder.
7. *Western washing buffer:* 10 mM Tris pH:7.5, 0.25 mM EDTA, 150 mM NaCl, 0.1% Tween-20.
8. *Western transfer buffer:* 14 g/L Glycine, 3 g/L Tris-base, 20% Methanol.
9. Nitrocellulose membrane.
10. LiCor secondary rabbit antibody.
11. LiCor instrument.

2.5 Mitochondrial Footprint Isolation

1. Acid Phenol:Chloroform:IAA (125:24:1), pH 4.5.
2. 100% Isopropanol.
3. 3 M NaOAc stock in nuclease-free water.
4. GenElute LPA Linear Polyacrylamide 25 mg/mL stock solution.
5. 70% EtOH stock in nuclease-free water.

2.6 Mitochondrial Footprint Size Purification

1. *2× Urea loading buffer*: 0.03% Urea, 6.5 mM EDTA, 1 mM Tris pH 7.5, and 0.6 mg/mL of bromophenol blue in nuclease-free water.
2. Low molecular weight DNA ladder.
3. 28 nt RNA oligo [5′AGU CAC UUA GCG AUG UAC ACU GAC UGU G/3Phos/].
4. 10× TBE buffer (0.89 M tris, 0.89 M borate, and 0.02 M EDTA).
5. 15% polyacrylamide TBE-Urea precast gels.
6. SYBR Gold stain.
7. Blue light cutting surface.
8. 18G needle.
9. CoStar 1× spin columns.
10. 100% Isopropanol.
11. 3 M NaOAc stock in nuclease-free water.

12. GenElute LPA Linear Polyacrylamide 25 mg/mL stock solution.
13. 70% EtOH stock in nuclease-free water.

2.7 Library Preparation

In order to monitor all steps of the library construction, use a purified RNA oligo of similar size, for example, 28 nucleotides (Table 1).

2.7.1 RNA Dephosphorylation

1. 10× T4 PNK buffer.
2. T4 PNK.

2.7.2 3′ End Linker Ligation

1. Linker N10: 5′ pre-adenylated, 3′ blocked at 1 μg/μL [/5rApp/**NNNNNNNNNN**CTGTAGGCACCATCAAT/3ddC/].
2. 50% PEG 8000.
3. 10× T4 RNL2 buffer.
4. T4 RNL2.

2.7.3 Purification of 3′ End Ligation Product

1. 100% Isopropanol.
2. GenElute LPA Linear Polyacrylamide 25 mg/mL stock solution.
3. 3 M NaOAc stock in nuclease-free water.
4. 70% EtOH stock in nuclease-free water.
5. 10% TBE-Urea precast gel.
6. SYBR Gold stain.
7. *2× Urea loading buffer*: 0.03% Urea, 6.5 mM EDTA, 1 mM Tris pH 7.5 and 0.6 mg/mL of bromophenol blue in nuclease-free water.
8. Low molecular weight DNA ladder.
9. Blue light cutting surface.
10. 10 mM Tris–HCl pH 8.0.

2.7.4 Reverse Transcription

1. 1.25 μM RT Random Primer [/5Phos/**NNNN**GATCGTCGGACTGTAGAACTCTGAACCTGTC/iSp18/CACTCA/iSp18/CAAGCAGAAGACGGCATACGAGATATTGATGGTGCCTACAG].
2. 5× First Strand (FS) buffer.
3. 10 mM dNTPs.
4. 0.1 M DTT.
5. SuperaseIn.
6. SuperScriptIII.
7. 1 N NaOH.

Table 1
Oligos sequences and application in protocol

Oligo	Sequence	Notes
oGAB11	5′ AGU CAC UUA GCG AUG UAC ACU GAC UGU G/3Phos/	RNA oligo used as control for library preparation.
N10 3′ Linker	/5rApp/NNNNNNNNNNCTGTAGGCACCATCAAT/3ddC/	DNA oligo 5′ pre-adenylated, 3′ blocked. Introduces 10 random bases (N) to the 3′ end.
RT 4N Primer	5Phos/NNNNGATCGTCGGACTGTAGAACTCTGAACCTGTC/iSp18/ CA CTCA/iSp18/CAAGCAGAAGACGGCATACGAGATATT GATGGTGCCTA CAG	DNA oligo for reverse transcription reaction. Introduces 4 random bases (N) to the 5′ end.
PCR Forward Primer	CAAGCAGAAGACGGCATACGAGATATTGATGG	DNA oligo for PCR amplification of libraries. Amplifying 5′ → 3′.
PCR Reverse Primer (Indexed)	AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACAC GTCTGAACTCCAGTCACNNNNNNCGACAGGTTCAGAGTTC	DNA oligo for PCR amplification of libraries. Amplifying 3′ → 5′ introducing Illumina index (N).
Custom Sequencing Primer	CGACAGGTTCAGAGTTCTACAGTCCGACGATC	DNA oligo for sequencing libraries.

2.7.5 Purification of Reverse Transcription Product

1. 100% Isopropanol.
2. GenElute LPA Linear Polyacrylamide 25 mg/mL stock solution.
3. 3 M NaOAc stock in nuclease-free water.
4. 70% EtOH stock in nuclease-free water.
5. 10% TBE-Urea precast gel.
6. SYBR Gold stain.
7. *2× Urea loading buffer*: 0.03% Urea, 6.5 mM EDTA, 1 mM Tris pH 7.5 and 0.6 mg/mL of bromophenol blue in nuclease-free water
8. Low molecular weight DNA ladder.
9. Blue light cutting surface.

2.7.6 Circularization

1. 10× CircLigase buffer.
2. 1 mM ATP.
3. 50 mM $MnCl_2$.
4. CircLigase enzyme.

2.7.7 PCR Amplification

1. 5× Phusion buffer.
2. 10 mM dNTPs.
3. 20 μM Forward Primer [CAAGCAGAAGACGGCATAC GAGATATTGATGG].
4. 20 μM Reverse Primer (*Illumina Index*) [AATGATACGGC-GACCACCGAGATCTACACGATCGGAAGAGCACAC GTCTGAACTCCAGTCAC**NNNNNN**CGACAGGTTC AGAGTTC].
5. 2 U/μL Phusion Polymerase.

2.7.8 PCR Product Purification

1. 8% TBE precast gel.
2. *10× DNA loading buffer:* 400 μL/mL of 100% Glycerol, 0.5% SDS, 10 mM EDTA pH 8 and 5 mg/mL of bromophenol blue.
3. 100% Isopropanol.
4. GenElute LPA Linear Polyacrylamide 25 mg/mL stock solution.
5. 3 M NaOAc stock in nuclease-free water.
6. 70% EtOH stock in nuclease-free water.
7. SYBR Gold stain.
8. 100 bp DNA ladder.
9. Blue light cutting surface.

2.8 Library Quality Controls and Quantitation

1. Qubit quantification reagent for high-sensitivity DNA.
2. BioA regents for high-sensitivity DNA.
3. Qubit instrument.
4. BioA instrument.

2.9 Library Sequencing

1. 100 μM Custom sequencing primer in nuclease-free water [CGACAGGTTCAGAGTTCTACAGTCCGACGATC].
2. Illumina platform sequencing [we used a NextSeq 500 instrument].

2.10 Data Analysis

1. High-performance computing cluster or equivalent computing power.
2. Programs installed: Python, R, cutadapt, STAR, samtools, jvarkit.

3 Methods

3.1 Cell Growth

Cell cultures should be grown in dishes to facilitate quick access during lysis. Plan to collect at least 5×10^6 cells for each sample to have enough material to perform mitoribosome profiling. Grow human cell lines in DMEM supplemented with 10% FBS. To prepare spike-in lysates, culture mice fibroblasts in the same way as human cells, but use 10% Cosmic serum in cultures instead of FBS. Plan to generate enough mouse lysate to spike in all samples of the experiment (95%:5%, human:mouse by A260 values). Grow all cultures in the absence of streptomycin or penicillin to avoid any antibiotic effects on the mitoribosome.

1. Grow cell cultures in 15 cm dishes containing 35 mL of FBS or Cosmic serum-supplemented medium.
2. Allow cells to reach 75–80% confluency in a 37 °C, 5% CO_2 incubator.

3.2 Cell Lysis and Sample Collection

When collecting cell samples for ribosome profiling it is critical to minimize the manipulation time and to keep the samples cold to avoid disrupting ribosome-RNA interactions. Collect and freeze human and mouse samples independently. They will be mixed before RNase digestion described in Subheading 3.3.

1. Lysis and sample collection can be done either in the main lab space or in a cell culture room hood, if a vacuum line is available. Place plate on ice, remove media using vacuum line, and quickly wash plate once with 10 mL ice-cold 1× PBS, pH 7.4. Remove as much liquid as possible.

2. Add 600 μL (*see* **Note 2**) of ice-cold lysis buffer, scrape cells with cell lifter, and transfer to a loose-fit glass/glass 5 mL dounce homogenizer. Dounce 5–6 times while on ice. Move homogenized lysates to 1.5 mL RNase-free tubes in either 450 μL (human), or 200 μL (mouse) aliquots, flash-freeze tubes in a styrofoam bucket containing liquid nitrogen. Move aliquots to −80°C freezer for storage (*see* **Note 3**).

3.3 RNase Digestion

Careful titration of RNase I treatment is necessary to balance between the enrichment of tight footprints and not disrupting the RNA-containing mitoribosomes. Mouse lysate is added to the human lysate prior to this step so they are processed in parallel. RNase I efficiency varies from brand to brand and between lots of the same brand, requiring its titration for the specific available enzyme and cell line used. The conditions presented here are specific for HeLa cell lysates digested with New England Biolabs RNase If enzyme.

1. Thaw human and mice whole cell lysates on ice.
2. Measure RNA concentration of lysates using NanoDrop. For each sample, use A260 estimates to mix human and mouse lysates in 95:5% (human: mouse) ratios (*see* **Note 4**).
3. To 450 μL of mouse-spiked human lysate, add RNase I so that the enzyme Units/A260 is 0.04 Units/ng/μL. Digest at room temperature for 30 min without rotation. To stop digestion, add 0.180 U/μL of SuperaseIn and place tube on ice (*see* **Note 5**).
4. Clarify lysates by centrifugation at 12,500 RCF for 5 min at 4 °C. Transfer clarified supernatant to a clean tube. Keep on ice. Use in Subheading 3.4. Save 15 μL of clarified lysate as input control for immunoblotting in Subheading 3.5.

3.4 Mitochondrial Ribosome Isolation

In mammalian cells, the sedimentation coefficient of the mitoribosome is significantly different from that of the cytoribosome allowing the use of sucrose gradients for purification. Using a linear gradient of wide range (10–50%), instead of a cushion, offers the resolution of all species of the mitoribosome: the small and large subunits, monosomes, and polysomes. Such separation is critical in avoiding unwanted RNA contaminants in the libraries.

3.4.1 Sucrose Gradient Sedimentation

1. Prepare enough 10% and 50% sucrose mixes to use 6 mL of each per gradient (*see* **Note 1**). Secure 12 mL ultracentrifuge tubes on a rack and layer 10% sucrose mix first at the bottom of the tubes. Using a long needle cannula, go to the bottom of the tube and place 6 mL of the 50% sucrose mix under the 10% layer, being careful not to introduce bubbles. Cap tubes using

Biocomp rubber stoppers, being careful to seal the tubes without any air or bubbles at the top.

2. Use Biocomp Sucrose Gradient Master instrument to mix gradients following a program to form linear sucrose concentration gradients from 10% to 50%. Fourteen cycles of time and angles:

   S01. 0:05/85.0/35

   S02. 0:01/77.0/00

   S03. 0:04/86.0/35

   S04. 0:01/77.0/00

   S05. 0:03/86.5/35

   S06. 0:01/77.0/00

   S07. 0:05/85.0/35

   S08. 0:01/77.0/00

   S09. 0:04/86.0/35

   S10. 0:01/77.0/00

   S11. 0:03/86.5/35

   S12. 0:01/77.0/00

   S13. 0:20/81.0/14

   S14. 0:07/86.0/20

3. Once ready, gradients can be used within 1 h of mixing, kept at 4 °C until samples are ready to load (*see* **Note 6**).
4. To load samples, uncap tubes and remove volume from top of gradient equal to volume of clarified lysate to be loaded (*see* **Note 7**). Carefully load up to 450 μL of sample with a 1 mL tip placed against the tube wall to avoid disturbing the gradient.
5. Centrifuge gradients using SW 41 Ti rotor in an ultracentrifuge at 40,000 RPM for 3 h at 4 °C. This time and speed will capture all mitoribosome species from the small subunit to polysomes.
6. Fractionate gradients using the automated Biocomp fractionator system into 12 equal-volume fractions. Cytosolic ribosomes are detected using a 254 nm UV filter, and traces are saved and recorded as csv files (*see* Fig. 2).
7. Save 40 μL of each fraction to be used in immunoblot experiments (*see* Subheading 3.4.2) (*see* **Note 8**). Freeze remainder of fractions at −80 °C until mitomonosome fraction has been confirmed by immunoblot analysis.

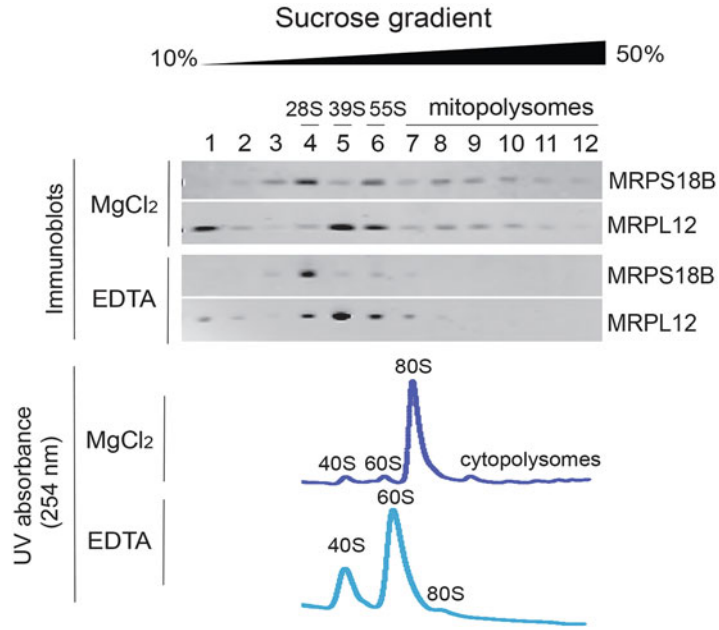

Fig. 2 Sedimentation of mito- and cytoribosomes in 10–50% linear sucrose gradients. HeLa S3 cells were lysed in the presence of either $MgCl_2$ or EDTA. Clarified lysates were loaded onto linear 10–50% sucrose gradients. Detection of mitoribosome (top panel) in each fraction was achieved by Western blots against proteins of the large (MRPL12) and small subunit (MRPS18B). We followed the sedimentation of cytoribosomes by their UV absorbance at 254 nM (bottom panel)

3.4.2 Mitochondrial Ribosome Visualization

Cytosolic ribosomes significantly outnumber mitoribosomes, making the detection of mitoribosomes in whole cell lysates challenging using UV absorbance. We used immunoblot analysis targeting both subunits of the mitoribosome to visualize mitoribosomes sedimentation in sucrose gradients and to monitor their integrity.

1. Prepare immunoblot samples by adding 10 μL of 5× loading buffer to the 40 μL sample saved in Subheading 3.4.1, **step 6**. Mix well and resolve on a 10% 15-well Bis-Tris polyacrylamide gel (*see* **Note 9**). Load 20 μL of sample/well and 5 μL of pre-stained protein ladder standard.
2. Run the gel until blue dye reaches the bottom, then transfer at 400 mA for 1 h and 15 min to a nitrocellulose membrane using western transfer buffer at 4 °C.
3. Block the nitrocellulose membrane in 5% skim milk powder in western washing buffer for 30 min.
4. Remove milk and add MRPL12 and MRPS18B antibodies each at 1:1000 v/v, diluted in 5% milk dissolved in western washing buffer. Incubate overnight at 4 °C on a shaking platform (*see* **Note 10**).

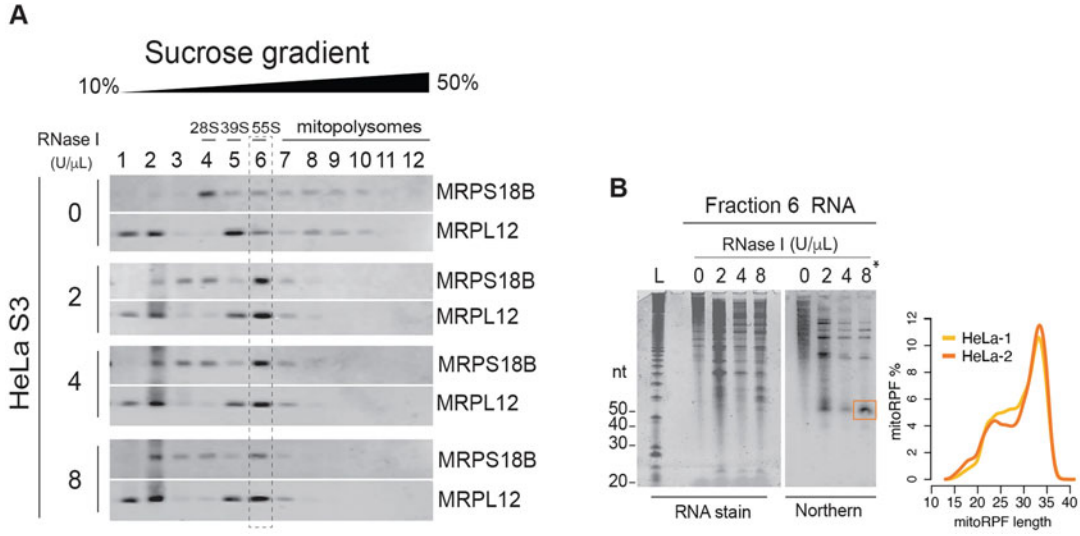

Fig. 3 Titration of RNase I to generate mitoribosome footprints. Increasing concentrations (0, 2, 4, and 8 Units/μL) of the nuclease were used to digest 450 μL of HeLa S3 lysate at room temperature for 30 min. Digested lysates were further clarified and loaded onto 10–50% linear sucrose gradients. (**a**) Detection of mitoribosomes in each fraction was achieved by Western blots against proteins of the large (MRPL12) and small subunit (MRPS18B). Dotted lined box indicates mitomonosome fraction. (**b**) RNA from the mitomonosome fraction (fraction 6) was further analyzed by Northern blot (bottom panel), showing a robust and compact footprint for the highest RNase I amount used (8 Units/μL), denoted with an (*) as the concentration used in subsequent sample generation. The mitoRPF length distribution from this sample (marked inside the box) and a replicate are shown to the right

5. Wash the membrane three times for 15 min at room temperature using western washing buffer. Add secondary LiCor antibody (rabbit-680), diluted to 1:10,000 volumes in 5% milk in western washing buffer. Incubate for 1 h at room temperature.
6. Remove the secondary antibody and wash the membrane twice for 15 min at room temperature with western washing buffer. Scan membrane using a LiCor instrument set to 700 nm wavelength (rabbit-680) (*see* Figs. 2 and 3a).

3.5 Mitochondrial RPF Isolation

MitoRPF isolation was performed exclusively from the identified mitomonosome fraction in Subheading 3.4. After sucrose gradient fractionation, the selected fraction can either be flash frozen in liquid nitrogen and stored at −80 °C for up to a week, or directly extracted for RNA isolation as explained below.

1. Mitoribosome footprints were recovered from the monosome-enriched fraction. To each sample, add equal volume of acid phenol/chloroform, pH 4.5. Vortex vigorously for 20 s and centrifuge at 4 °C for 10 min at 15,000 RPM. Transfer aqueous top layer to a clean RNase-free microcentrifuge tube.

2. Precipitate RNA using NaOAc at a final concentration of 0.3 M, 1.25 volumes of 100% ice-cold isopropanol, and 2 μL/mL of LPA. Place on dry ice for 15 min. [STOP POINT] (*see* **Note 11**). Centrifuge samples at 15,000 RPM, 4 °C for 20 min. Wash pellets once with room temperature 70% EtOH and resuspend in 20 μL of RNase-free water. RNA can be stored at −80°C or proceed to Subheading 3.6.

3.6 MitoRPF Gel Purification

The next step is to resolve the mitomonosome RNA by polyacrylamide gel electrophoresis and purify the size range of interest (25–40 nt). Prior to this step, use 5 μL of the RNA (isolated in Subheading 3.5) to assess mitoRPF size by Northern blot analysis (*see* Fig. 3b) as described in [10] (*see* **Note 5**). Visualizing mitoRFPs at this step will assess the effectiveness of the RNase treatment and ensure an accurate RNA size range is included in the library preparation. MitoRPF length (*see* Fig. 3b) will be reassessed bioinformatically after sequencing the libraries (*see* Subheading 3.10).

1. Combine 15 μL of RNA from Subheading 3.5 with 20 μL 2× Urea RNA loading buffer.
2. Prepare 0.5 μg of low molecular weight DNA ladder in 20 μL of 1× Urea RNA loading buffer.
3. Denature samples and ladder 90 s at 80 °C, immediately followed by 90 s on ice.
4. Pre-run a 15% TBE-Urea polyacrylamide gel in 1× RNase-free TBE buffer 15 min at 200 V (*see* **Note 12**). Load samples and 10 μL of ladder onto gel and run for 1.5 h at 200 V or until the front of the gel reaches bottom.
5. Stain gel with SYBR Gold (1:10,000 in RNase-free 1× TBE) for 2 min with shaking at room temperature. Rinse gel once in RNase-free water, place in a sheet protector, and image on Typhoon scanner. Use these scanning settings: 520 BP emission filter, blue (488) laser, and PMT voltage 400–600. If a Typhoon instrument is not available, image gel using UV transilluminator.
6. Prepare microcentrifuge tubes for crushing the gel slices. Pierce the bottom of a 0.5 mL tube using a 18G needle and nest it inside a 1.5 mL tube.
7. Place stained gel on a blue light cutting platform and excise sizes from 25 to 40 nt (*see* Fig. 4). Move gel pieces to the nested tube setup. Close smaller tube and leave bigger tube open. Centrifuge tubes at 14,000 RPM for 2 min. Gel slurry will be collected at the bottom of the 1.5 mL tube.
8. Remove the smaller tube and add 400 μL of RNase-free water to the gel slurry. Incubate at 70 °C for 15 min.

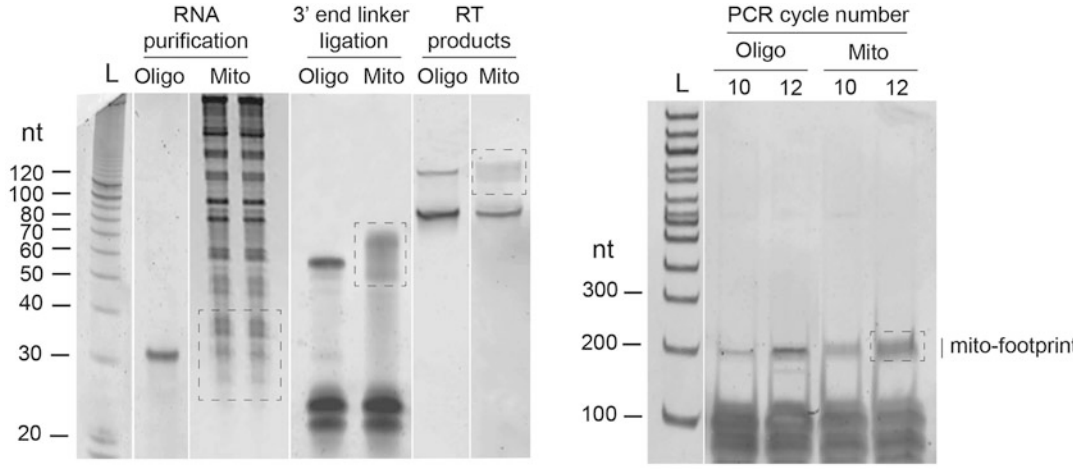

Fig. 4 Effect of using translation inhibitors in ribosome profiling. Comparison of relative synthesis rate values (tpm) for mtDNA-encoded OXPHOS subunits in cells exposed or not to ribosome inhibitors. Pearson correlation, r. HeLa cells were treated with cycloheximide (CHX, 100 μg/mL) and chloramphenicol (CAP, 100 μg/mL) for 15 and 5 min, respectively, to stall cytosolic and mitochondrial translation. After treatment, cells were washed with 1× PBS, pH 7.4 and subjected to lysis using our mitoribosome profiling conditions

9. Move gel slurry solution to a CoStar Spin column using a wide-bore tip and centrifuge at 14,000 RPM for 2 min at room temperature.
10. Move flow through to a clean tube and precipitate recovered RNA using 0.3 M NaOAc, 1.25 volumes of 100% cold isopropanol and 2 μL/mL of LPA. Incubate on dry ice for 15 min and centrifuge at 15,000 RPM for 20 min at 4 °C. [STOP POINT] (*see* **Note 11**).
11. Wash pellets with room temperature 70% EtOH and resuspend in 5 μL of RNase-free water.

3.7 Library Preparation

Libraries are prepared as described in [3], with a few modifications. Using only the RNA from the mitomonosome fraction provides a more purified sample, avoiding the need for rRNA depletion. We also introduce ten randomized nucleotides to the linker ligation at the 3′ end and 4 to the 5′ end. All intermediate products in the library preparation are visualized and purified using gels in order to monitor and quality control every step of the library generation process.

3.7.1 RNA Dephosphorylation

1. Denature the 5 μL of RNA from Subheading 3.5 for 90 s at 80 °C and immediately place sample on ice for 90 s.
2. Add 4.5 μl PNK mix: 0.95 μL 10× T4 PNK buffer, 2.55 μL RNase-free water, 1 μL T4 PNK. Incubate at 37 °C, 1 h.
3. Heat-inactivate T4 PNK by incubating at 70 °C for 10 min. Spin tube to collect possible condensation.

3.7.2 3′ End Linker Ligation

1. To the dephosphorylated sample, add 0.5 μL of N10 3′ Linker (Table 1) at 1 μg/μL for 0.5 μg, 85 pmol final concentration (*see* **Note 13**). Denature at 80 °C for 90 s, followed by an incubation on ice for 90 s.
2. Add 10 μL ligation mix: 8 μL 50% PEG 80008000, 1 μL 10× T4 RNA ligase buffer, 1 μL T4 RNL2, truncated (*see* **Note 14**). Incubate at room temperature for 3 h.
3. Add 400 μL of RNase-free water, and follow instructions for RNA precipitation explained in Subheading 3.6, **step 10**.

3.7.3 Purification of 3′ End Ligation Product

1. Resuspend pellets in 10 μL 1× Urea RNA load buffer.
2. Prepare 0.3 μg of low molecular weight DNA ladder in a final volume of 10 μL in 1× urea RNA loading buffer.
3. Denature samples and ladder at 80 °C for 90 s, followed by ice for 90 s.
4. Load samples and ladder on a 10% TBE-Urea polyacrylamide gel and run for 1 h at 200 V or until blue dye reaches the bottom of the gel.
5. Stain and image gel as described above in Subheading 3.6, **step 5**.
6. Excise ligated product (*see* Fig. 4) from 50 to 70 nt and perform gel extraction and isopropanol precipitation as explained in Subheading 3.6, **steps 6–11**. [STOP POINT] (*see* **Note 11**).
7. Resuspend ligated RNA in 10 L of 10 mM Tris, pH 8.0

3.7.4 Reverse Transcription

1. Transfer resuspended ligated RNA to a PCR tube. All subsequent incubation steps should be performed in a thermocycler.
2. Add 2 μL of 1.25 μM RT 4 N primer (Table 1).
3. Denature for 90 s at 80 °C and immediately place on ice for 90 s.
4. Add 8 μL reverse transcription mix: 4 μL 5× FS buffer, 1 μL 10 mM dNTPs, 1 μL 0.1 M DTT, 1 μL SuperaseIn, 1 μL Superscript III. Mix well. Incubate 30 min at 48 °C.
5. Add 2.2 μL of 1 N freshly diluted sodium hydroxide and incubate 20 min at 98 °C to degrade RNA.
6. Transfer to a 1.5 mL microcentrifuge tube and add 400 μL water. Precipitate sample as described above in Subheading 3.6, **step 10**.

3.7.5 Purification of Reverse Transcription Product

1. Resuspend pellet in 10 μL 1× Urea RNA load buffer.
2. Prepare 0.3 μg low molecular weight DNA ladder in a final volume of 10 μL 1× Urea RNA load buffer. Denature sample

and ladder and load on a 10% TBE-Urea polyacrylamide gel. Run gel for 1 h at 200 V or until blue dye reaches the bottom of the gel.

3. Stain and image gel as described above in Subheading 3.6, **step 5**.
4. Excise ligated product (*see* Fig. 4) from 90 to 110 nt and perform gel extraction and isopropanol precipitation as explained in Subheading 3.6, **steps 6–11**. [STOP POINT]. (*see* **Note 15**).
5. Resuspend purified RT product in 5 uL of nuclease-free water.

3.7.6 Circularization

1. To resuspended RT product, add 15 μL circularization mix: 10 μL water, 2 μL 10× circularization buffer, 1 μL 1 mM ATP, 1 μL 50 mM manganese chloride, 1 μL CircLigase.
2. Incubate 1 h at 60 °C.
3. Heat-inactivate CircLigase by incubating 10 min at 80 °C. Store circles at −20 °C.

3.7.7 PCR Amplification of Libraries

PCR Cycle Number Determination

1. Prepare a master mix reaction to test different PCR cycle numbers: Combine 5 μL of circles with 78.5 μL PCR mix: 56.3 μL water, 16.7 μL 5× Phusion HF buffer, 1.7 μL 10 mM dNTPs, 1.5 μL 20 μM oSMD2 forward primer (Table 1), 1.5 μL 20 μM indexed primer (Table 1), 0.8 μL 2 U/μL Phusion polymerase (*see* **Note 16**).
2. Aliquot 16.7 μL reaction mixture into each of five PCR tubes.
3. Program thermocycler 30 s at 98 °C and 14 cycles of: 15 s at 98 °C, 15 s at 65 °C, 7 s at 72 °C.
4. Remove samples from thermocycler during elongation step (72 °C) after 10 and 12 cycles (*see* Fig. 4).
5. To each sample, add 2.0 μL 10× DNA loading buffer.
6. Prepare 0.3 μg of 100 bp-DNA ladder in a final volume of 20 μL 1× DNA load buffer. Use 10 μL/gel.
7. Do not heat-denature samples, but load directly on a 8% TBE non-denaturing polyacrylamide gel. Run gel in 1× TBE for 45 min at 180 V. Pre-running the non-denaturing gel is optional.
8. Stain and image gel as previously described (Subheading 3.6, **step 5**).

PCR Amplification with Determined Cycle Number

1. Prepare PCR reaction mix as described in Subheading 3.7.7.1. Make enough master mix/sample to amplify eight reactions of 16.7 μL each.
2. Aliquot 16.7 μL reaction mixture into PCR tubes.

3. Set thermocycler program for 30 s at 98 °C for the number of cycles determined in Subheading 3.7.7.1 of: 15 s at 98 °C, 15 s at 65 °C, 7 s at 72 °C.

3.7.8 PCR Product Purification

1. Pool all reactions for each sample and resolve them in non-denaturing conditions as explained in Subheading 3.7.7.1
2. Excise amplified PCR product at around 180–190 nt, avoiding any lower product band resulting from unextended reverse transcription primer (*see* Fig. 4).
3. Perform overnight gel extraction: Place gel pieces in 1.5 mL microcentrifuge tube and add 650 μL of DNA gel extraction buffer. Incubate overnight at room temperature, rotating end over end.
4. Move liquid to a CoStar Spin column and centrifuge for 2 min at 14,000 RPM to remove any gel residue.
5. Precipitate libraries by adding 2 μL/mL of LPA and 800 μL of ice-cold 100% isopropanol. Incubate on dry ice for 15 min and spin at 15,000 RPM at 4 °C for 20 min. Wash pellet once with room temperature 70% EtOH. Resuspend pellets in 15 μL of 10 mM Tris, pH:8.5.

3.8 PCR Product Quality Controls and Quantitation

Assess library concentration and quality using Qubit Fluorometric Quantitation dsDNA kit (high sensitivity), and Bioanalyzer DNA high-sensitivity chip according to manufacturer's instructions.

3.9 Sequencing

When submitting samples for sequencing, supply custom "Read 1" sequencing primer: oNTI202 (Table 1).

3.10 Data Analysis

Human mitoRPFs are typically 30–34 nucleotides. The UMI is 14 nucleotides, so 60-cycle reads are sufficient for covering these plus enough of the 3′ adapter for identification and removal. For robust downstream analyses, plan for at least 30–50 million raw reads, although results vary depending on the cell type. Cytosolic rRNA typically composes 50–70% of all reads and mitochondrial rRNA around 10%. In mitochondria-rich cell types however the mitochondrial:cytosolic rRNA ratio may be increased and vice versa for mitochondria-poor cell types. Mitochondrial mRNA-mapping reads (the mitoRPFs) should ultimately make up 3–10% of the raw reads. PCR duplicates should be removed using the UMI and not a prebuilt tool that uses only alignment positions to identify them.

For more details, see https://github.com/churchmanlab/human-mitoribosome-profiling

3.10.1 Raw FASTQ Files Initial Processing

1. Remove adapter sequence (e.g., with Cutadapt https://github.com/marcelm/cutadapt). Discard reads shorter than 25 nucleotides after adapter removal. The 14-nt UMI will be

removed in the next step leaving a minimum of 11-nt RPFs. This short minimum length is used to allow retention of potential halfmers resulting from translation initiation on leaderless mRNAs. Discard reads in which the adapter was not identified because the UMI cannot be identified in these cases.

2. Remove four nucleotides from the 5′ end and ten nucleotides from the 3′ end. These together are the UMI, which is to be associated with the sequence identifier.
3. Remove the reads aligning to cytosolic and mitochondrial rRNAs.
4. Map the remaining reads with a splice aware alignment tool, for example, STAR (https://github.com/alexdobin/STAR) to the reference genome that includes nuclear chromosomes and the mitochondrial genome (*see* **Note 17**). If mouse lysate was added for spike-in use, the mouse genome should be concatenated with the human genome for alignment in order to get the most accurate results. However, spike-in read counts will be determined from a separate alignment step (*see* Subheading 3.10.1, **step 8**).
5. Remove true PCR duplicates by collapsing reads that have identical mapping coordinates, sequence, and UMI to a single occurrence.
6. Filter the deduplicated BAM file using samtools (https://github.com/samtools) to keep only mitochondrial mRNA mapping reads. The resulting BAM file will be the input for the following section.
7. In parallel to **steps 4–6**, determine the spike-in normalization factor: Filter the output of **step 3** for a minimum read length of 20 nucleotides.
8. Align to mouse mitochondrial mRNAs (not the entire mouse mitochondrial genome) allowing 0 mismatches. Either allow soft clipping or trim the 5′ terminal nucleotide which is often added, untemplated, by reverse transcriptase during library preparation.
9. Remove true PCR duplicates as in **step 5**.
10. The resulting read count is the spike-in normalization factor.

3.10.2 Transform the Read Signal to Ribosome A Site Positions

This section describes how to transform the read signal to ribosome A site positions (*see* **Note 18**). Only three mitochondrial genes have 5′ regions upstream of the start codon that are long enough to observe full-length mitoRPFs of initiating mitoribosomes. These genes are MT-ATP6 and MT-ND4, which both overlap with their upstream genes MT-ATP8 and MT-ND4L, respectively, in bicistronic transcripts. The third is MT-CO3, which is immediately downstream of MT-ATP6 and is inefficiently processed, leaving a

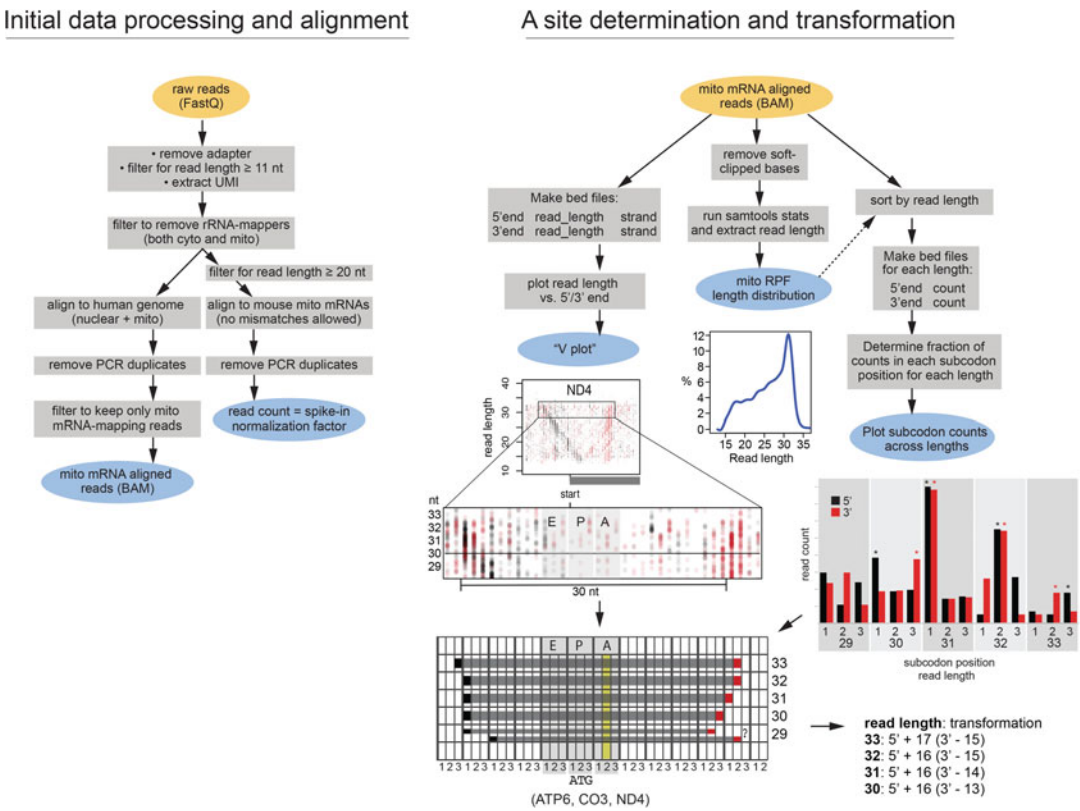

Fig. 5 Library preparation for mitoribosome profiling. All steps described in Subheading 3.7, including: mitoRFPs gel purification, 3′ end linker ligation, Reverse Transcription (RT) reaction and PCR amplification. Dotted lined boxes indicate areas in the gel that were included for product isolation. RNA and DNA products were run in either denaturing TBE-Urea acrylamide gels (mitoRFPs isolation, linker ligation, RT product), or non-denaturing TBE acrylamide gels (PCR amplification product). RNA and DNA products were visualized using SYBR Gold staining and their size estimated using a 10 bp DNA ladder (L). We included RNA oGAB11 (Oligo) (Table 1) as a technical control through all steps of the library preparation process to monitor the quality of the mitoRPF products (Mito)

tricistronic transcript (MT-ATP8/ATP6/CO3). These start codons are used to set the approximate register for the offset from the 5′ or 3′ end of reads to the A site. To determine the exact offset, the most frequent subcodon position of 5′ and 3′ ends for each read length is calculated from across all open reading frames. Notably, because trimming can occur from both ends, reads that are not the full length of 30–34 nucleotides will have an ambiguous register and their A site offset cannot be determined (e.g., 29 nt reads in Fig. 5). The input file for the following steps is the BAM file from **step 6** above: deduplicated human mitochondrial mRNA mappers.

1. First, plot the mitoRPF read length distribution. For an accurate output, soft-clipped bases must first be removed (*see* note in Subheading 3.10.1, **step 8**), which can be done using jvarkit (https://github.com/lindenb/jvarkit/blob/master/src/main/java/com/github/lindenb/jvarkit/tools/biostar/Biostar84452.java). Samtools stats will then output the read length distribution directly from the BAM file. See README.md and scripts at https://github.com/churchmanlab/human-mitoribosome-profiling/tree/main/1_AlignData.
2. Next make "V plots," which plot read length versus genomic position, to visualize mitoRPF 5′ and 3′ ends in relation to start codons. To do this, make two 3-column files: (1) 5′ position, read length, strand and (2) 3′ position, read length, strand. Again soft-clipped bases are ignored, unless they result from poly(A) tails. See README.md and scripts at https://github.com/churchmanlab/human-mitoribosome-profiling/tree/main/2_MakeLengthBeds.
3. Finally find the most frequent subcodon position (1st, 2nd, or 3rd) for both 5′ and 3′ ends of full-length reads. This is done individually for each read length. Make individual SAM files for each read length in the range of full-length as determined in **step 1** above (e.g., 29–33 nucleotides). From each of these make two 2-column files: (1) 5′ position, read count and (2) 3′ position, read count. Using these, determine the number of reads in each subcodon position across all open reading frames. See README.md and scripts at https://github.com/churchmanlab/human-mitoribosome-profiling/tree/main/3_CountFramePerLength.
4. Use the V plot at start codons and subcodon counts across lengths to determine the offset to the A site for each read length, as shown in Fig. 5. For example, take 30 nt reads (black horizontal line on the V plot in Fig. 5; also see the scale line below). The darkest red region (3′ ends) on this line is 16 nt downstream of the middle of the start codon, which is in the P site of an initiating ribosome. Now, looking at the subcodon position bar plot, we see that accordingly, 3′ ends of 30 nt reads are most frequently at the 3rd subcodon position (red asterisk) throughout the genome. The same process can be done using the 5′ ends of 30 nt reads and then for other read lengths. Occasionally, the precise positioning will be ambiguous on the V plot, which only represents a single locus. Therefore, it is important to always corroborate offsets using the subcodon bar plot, which uses data from across the entire transcriptome. Offsets can be taken from either the 5′ end or the 3′ end. Read signals are assigned to the center of the A site (subcodon position 2) so that even with one nucleotide of error

either way reads will still be assigned to the correct codon. To carry out A site transformation after determining offsets, see README.md and scripts at https://github.com/churchmanlab/human-mitoribosome-profiling/tree/main/4_AsiteTransformation.

4 Notes

1. $MgCl_2$ can be switched with 5 mM EDTA to assess polysome dissociation and ribosome subunit separation in sucrose gradients (*see* Fig. 2). Addition of antibiotics to buffers is optional. We did not detect differences in mitomonosome stability when omitting chloramphenicol and/or cycloheximide (*see* Fig. 6).
2. Make more lysis buffer than needed for volume adjustments. Adjust lysis buffer volume to RNA cell content. Use 600 µL of buffer for human fibroblasts and myoblasts, but 1 mL for HEK293T and HeLa S3 cells.
3. Cell lysates can be stored at −80 °C for up to 3–4 months. Do not freeze and thaw multiple times.
4. Mix enough sample volume to dedicate 450 µL of lysate for mitoribosome profiling and 400 µL for RNA-seq for RNA abundance control, so that both experiments share the same input source.
5. Enzyme concentration was estimated for the specific RNase brand and titrated for each cell line used. Footprint size after RNase digestion was assessed using Northern blot analysis as previously described [10]. Example: HeLa cells required 8 Units/µL of enzyme to digest 450 µL of lysate at 200 ng/µL concentration estimated by absorbance at 260 nm, while human fibroblasts required 4.4 Units/µL.

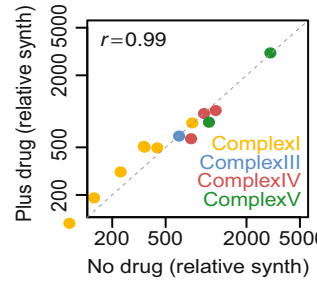

Fig. 6 Flow chart of the computational steps for the analysis of human mitoribosome profiling. Inputs and outputs are highlighted, and sample plots are shown to illustrate the process of determining the A site transformation, which can be done using either the 5′ or the 3′ read end

6. Keep mixes at room temperature during gradient mixing, but cool gradients afterwards prior to use.
7. Removing a small volume from the top of the gradient will prevent overflow during sample fractionation and will not significantly affect the resolution of the gradient.
8. With the time and speed parameters used above, the mitomonosome peak is expected in fraction 6. Depending on sample volume loaded, the mitomonosome may also be found in fraction 5, which makes immunoblotting a necessary step for accurate monosome identification.
9. Do not boil western samples before running.
10. MRPL12 running size is ~12 kDa, while MRPS18B is ~21 kDa, making their separation possible using the above conditions. Both antibodies show minimal background signal, which allows for their simultaneous use on the same membrane.
11. Store RNA at −80 °C in alcohol prior to resuspending.
12. Clear urea from wells with a syringe and needle before the pre-run and immediately before loading the samples. Load 20 μL/well and leave one empty well in between samples to avoid cross-contamination. To fit this volume, use 1.5 mm thick gels, or split the volume to fit 1 mm gels.
13. Linker should be present in at least fivefold molar excess compared to substrate RNA.
14. PEG 8000 is very viscous and care must be taken in preparing this reaction. It is advisable to prepare enough mix for at least one extra reaction.
15. RT sample can be stored in precipitation mix for weeks at −80 °C before moving to the next step. If the product band runs too close to the primer band, the sample will need to be repurified in a second gel run.
16. Perform PCR test with the index primer that will be used for each library.
17. It is important to allow alignment of reads with multiple mapping positions and retain them for downstream processing because of nuclear mitochondrial DNA segments present in the nuclear genome.
18. Compute the A-site offsets for each experiment. While each cell line generally has a consistent offset, there are small differences between cell lines, and differences in RNase treatment can have an effect.

References

1. Elson JL, Smith PM, Greaves LC et al (2015) The presence of highly disruptive 16S rRNA mutations in clinical samples indicates a wider role for mutations of the mitochondrial ribosome in human disease. Mitochondrion 25:17–27
2. Yarham JW, Elson JL, Blakely EL et al (2010) Mitochondrial tRNA mutations and disease. Wiley Interdiscip Rev RNA 1:304–324
3. Ingolia NT, Brar GA, Rouskin S et al (2012) The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. Nat Protoc 7:1534–1550
4. Amunts A, Brown A, Toots J et al (2015) Ribosome. The structure of the human mitochondrial ribosome. Science 348:95–98
5. Rooijers K, Loayza-Puch F, Nijtmans LG, Agami R (2013) Ribosome profiling reveals features of normal and disease-associated mitochondrial translation. Nat Commun 4:1–8
6. Pearce SF, Rorbach J, Van Haute L et al (2017) Maturation of selected human mitochondrial tRNAs requires deadenylation. Elife:6. https://doi.org/10.7554/eLife.27596
7. Pearce SF, Cipullo M, Chung B et al (2021) Mitoribosome profiling from human cell culture: a high resolution view of mitochondrial translation. Methods Mol Biol 2192:183–196
8. Soto I, Couvillion M, McShane E et al (2021) Balanced mitochondrial and cytosolic translatomes underlie the biogenesis of human respiratory complexes. bioRxiv 2021.05.31.446345
9. Englmeier R, Pfeffer S, Förster F (2017) Structure of the human mitochondrial ribosome studied in situ by cryoelectron tomography. Structure 25:1574–1581.e2
10. Couvillion MT, Churchman LS (2017) Mitochondrial ribosome (mitoribosome) profiling for monitoring mitochondrial translation in vivo. Curr Protoc Mol Biol 119: 4.28.1–4.28.25