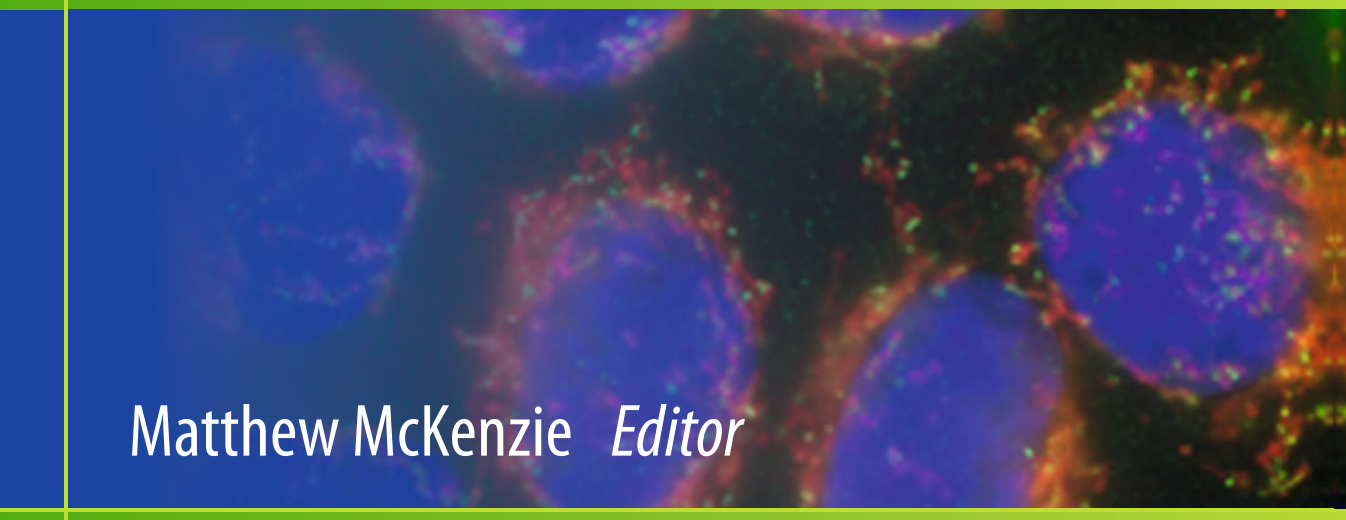
Methods in Molecular Biology 1351

Springer Protocols



Matthew McKenzie *Editor*

Mitochondrial DNA

Methods and Protocols

Third Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Third Edition

Edited by

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Preface

Since the publication of the second edition of *Mitochondrial DNA*: *Methods and Protocols*, major technological advances have been made in high-throughput DNA sequencing, high-resolution cellular imaging, and the use of targeted nucleases for genetic manipulation. These new scientific tools have revolutionized the study of human genetics and biology and apply equally to the investigation of the mitochondrial genome. This has allowed researchers to gain new insights into how mitochondrial DNA modulates mitochondrial metabolism and also the association between mitochondrial DNA variation and human disease.

This third edition of *Mitochondrial DNA*: *Methods and Protocols* describes these new methodologies for mitochondrial DNA analysis and, combined with well-established protocols that are considered the gold standard in the field, comprises a compendium of detailed methods for the study of mitochondrial DNA biology. The first part describes protocols for detecting mutations in mitochondrial DNA by next-generation massive parallel sequencing as well as techniques to assess mitochondrial DNA damage. The second part describes the visualization of mitochondrial DNA in situ and the detection of mitochondrial DNA nucleoids within the mitochondria. The third part describes methods for analyzing mitochondrial DNA replication, mitochondrial DNA-encoded protein translation, and mitochondrial DNA copy number. The fourth part describes the latest technology for modifying the mitochondrial genome, while the fifth part describes how to purify proteins involved in the replication and transcription of mitochondrial DNA.

These protocols will prove highly useful, not only for mitochondrial researchers but also for scientists studying human diseases where mitochondrial DNA variation has been recognized as an important pathogenic factor, including cancer and neurodegeneration. As such, this book will be a valuable addition to the *Mitochondrial DNA*: *Methods and Protocols* is the series title, and I would like to thank all of the authors for their excellent contributions to this publication.

Clayton, VIC, Melbourne, Australia ***Matthew McKenzie***

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Part I

Detection of Mitochondrial DNA Mutations and Mitochondrial DNA Damage

Chapter 1

Comprehensive Mitochondrial Genome Analysis by Massively Parallel Sequencing

Meagan E. Palculict, Victor Wei Zhang, Lee-Jun Wong, and Jing Wang

Abstract

Next-generation sequencing (NGS) based on massively parallel sequencing (MPS) of the entire 16,569 bp mitochondrial genome generates thousands of reads for each nucleotide position. The high-throughput sequence data generated allow the detection of mitochondrial DNA (mtDNA) point mutations and deletions with the ability to accurately quantify the mtDNA point mutation heteroplasmy and to determine the deletion breakpoints. In addition, this method is particularly sensitive for the detection of low-level mtDNA large deletions and multiple deletions. It is by far the most powerful tool for molecular diagnosis of mtDNA disorders.

Key words Mitochondrial whole genome, Next-generation sequencing, Massively parallel sequencing, mtDNA mutation heteroplasmy, mtDNA deletions

1 Introduction

Mitochondrial diseases are a group of clinically and genetically heterogeneous disorders with variable penetrance, expressivity, and different age of onset. Most disease-causing mtDNA mutations are heteroplasmic. The degree of mutant heteroplasmy varies in different tissues [1, 2]. Thus, the determination of mutant loads in affected tissues is important in making the diagnosis and correlating clinical phenotype. Current molecular analyses require multiple different and complementary methods, including Sanger sequencing, qPCR, Southern blot or array CGH for the detection and quantification of point mutations, large mtDNA deletion and multiple deletions, as well as mtDNA depletion. These approaches are labor-intensive, time-consuming, and have limitations in terms of accuracy and sensitivity.

The NGS method described here provides uniform coverage of each of the 16,569 bases of the mitochondrial genome at over

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20,000-fold. The deep coverage allows not only the detection of nucleotide changes, but also the degree of heteroplasmy at every single base [3, 4]. Moreover, this test is able to simultaneously detect small insertion/deletions (indels) and large deletions, and to map exact deletion breakpoints. This method demonstrates superior sensitivity and specificity of base calling with quantitative information when compared to the gold standard Sanger sequencing. In summary, the "deep" sequencing approach provides a one-step comprehensive molecular analysis for patients with suspicion of mitochondrial DNA diseases in a timely, accurate, and cost-effective manner.

2 Materials

2.1 Long-Range (LR) PCR

1. *TaKaRa LA Taq* DNA Polymerase, Hot Start Version 5 U/μL.
2. 10× LA PCR Buffer II: 25 mM Mg^{2+}, dNTP Mixture (2.5 mM each dNTP).
3. Nuclease-free water (ddH_2O).
4. LR PCR primers (*see* **Note 1**).
5. TE buffer: 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0.
6. Extracted total DNA (*see* **Note 2**).
7. Strip tubes, 0.2 ml with caps, 8 or 12 tubes per strip.
8. PCR Thermocycler.
9. Cool Plate.

2.2 Gel Electrophoresis

1. Agarose.
2. Agarose gel electrophoresis buffer (1× TAE): 40 mM Tris–acetate, 1 mM EDTA pH 8.0.
3. 1 % Ethidium Bromide (EtBr) Solution.
4. Horizontal electrophoresis apparatus with chambers and combs.
5. Microwave oven.
6. DNA marker: λ DNA-*Hind*III Digest (500 μg/mL).
7. Power supply device for gel electrophoresis.
8. 2× Agarose gel loading dye: 0.125 % bromophenol blue, 0.125 % xylene cyanol, and 7.5 % Ficoll 400.

2.3 DNA Purification

1. Ampure Beads (Beckman Coulter, Brea, CA, USA).
2. 100 % Ethanol (EtOH).
3. Nuclease-free water (ddH_2O).
4. Magnetic Stand.
5. 1.5 ml Eppendorf LoBind® tubes.
6. Heat Block.

2.4 DNA Quantification

1. Nano Drop Spectrophotometer.
2. Qubit 2.0 Fluorometer.
3. dsDNA HS Assay Kit (including Dilution buffer, Pre-diluted DNA standards, and Concentrated assay reagent).
4. Qubit Assay Tubes.
5. 2100 B BioAnalyzer and desktop bundle.
6. Agilent DNA 1000 Kit.
7. 7900HT Fast Real-Time PCR System.
8. Complete Library Quantification Kit: DNA Standards 1–6 (80 μL each, standard 1 is 20 pM, standard 2 is 2 pM, standard 3 is 0.2 pM, standard 4 is 0.02 pM, standard 5 is 0.002 pM, standard 6 is 0.0002 pM), 10× Primer Premix (1 mL), 2× KAPA SYBR FAST Universal (5 mL), and 50× ROX High/Low (200 μL each) (Illumina/Universal).
9. Nuclease-free water (ddH_2O).
10. 96-well Low Profile PCR microplate.
11. Heat-Resistant Films.

2.5 DNA Fragmentation

1. Covaris E220 with Conditioning System.
2. Cooling system.
3. Crimp or snap cap Covaris microTUBEs (130 μL).

2.6 DNA End Repair

1. NEBNext® End Repair Module: NEBNext End Repair Enzyme Mix, 10× NEBNext End Repair Reaction Buffer.
2. Nuclease-free water (ddH_2O).
3. Strip tubes, 0.2 mL with caps, 8 or 12 tubes per strip.
4. PCR Thermocycler.

2.7 DNA Adenylation

1. NEBNext® dA-Tailing Module: Klenow Fragment ($3' \rightarrow 5'$ exo^-), 10× NEBNext dA-Tailing Reaction Buffer.
2. Strip tubes, 0.2 mL with caps, 8 or 12 tubes per strip.
3. PCR Thermocycler.

2.8 DNA Adapter Annealing

1. Faststart Taq DNA Polymerase 5 U/μL kit: Faststart Taq Polymerase 5 U/μL, 10× PCR reaction buffer + 20 mM $MgCl_2$, 10× PCR reaction buffer with no $MgCl_2$, 25 mM $MgCl_2$, 5× GC-rich solution.
2. Multiplexing Adapter Forward and Reverse (100 μM) (*see* **Note 3**).
3. Nuclease-free water (ddH_2O).
4. Strip tubes, 0.2 ml with caps, 8 or 12 tubes per strip.
5. PCR Thermocycler.

2.9 DNA Ligation

1. NEBNext® Quick Ligation Module: Quick T4 DNA Ligase, 5× NEBNext Quick Ligation Reaction Buffer.
2. Index pair end adapter (InPE) mix 15 μM (*see* **Note 3**).
3. Strip tubes, 0.2 mL with caps, 8 or 12 tubes per strip.
4. PCR Thermocycler.

2.10 Enrichment PCR

1. Herculase II Fusion Enzyme with dNTPs Combo: Herculase II Fusion DNA Polymerase, 5× Herculase II Reaction Buffer, 25 mM each dNTPs mix.
2. Nuclease-free water (ddH_2O).
3. Indexing Primer (Reverse) 25 μM (Table 1).
4. InPE 1.0 (Forward) 25 μM (*see* **Note 4**).
5. Strip tubes, 0.2 mL with caps, 8 or 12 tubes per strip.
6. PCR Thermocycler.

3 Methods

3.1 LR PCR, Analysis and Purification

1. Set up two 50 μL reactions for each DNA sample:
   0.3 μL of 5 U/μL TaKaRa LA HS Taq, final 0.03 U.
   0.5 μL of 10× LA PCR Buffer II (with Mg^{2+}), final 1×.
   7 μL of 2.5 mM dNTP, final 0.35 mM.
   2 μL of 2.5 μM forward primer, final 0.1 μM.
   2 μL of 2.5 μM reverse primer, final 0.1 μM.
   2 μL of 50 ng/μL total extracted blood DNA solution, final 100 ng (if muscle or liver DNA, use total 20 ng) (*see* **Note 5**).
   31.7 μL of ddH_2O to a final volume of 50 μL.
2. LP PCR cycling conditions:

Block:	95 °C, 2 min	
	95 °C, 20 s 68 °C, 18 min	× 30 cycles
	72 °C, 20 min	
	4 °C, hold	

3. Run 2 μL samples on 1 % agarose gel. Use 1 μg NEB λ DNA-HindIII Digest marker (500 μg/mL). Run electrophoresis at 100 V for 90 min. Save gel picture (Fig. 1).
4. Purify the remaining 96 μL (by combining two reactions of the same sample) of LR PCR products that show a proper band (the intact circular mtDNA should give the band at 16.6 kb)

Table 1
Primer sequences for indexes L01-L12 (NEBNext Index 1–12 for Illumina)

index #	Index primers	For demultiplexing#
Index L01	CAAGCAGAAGACGGCATACGAGAT TGTTGACT GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	AGTCAACA
Index L02	CAAGCAGAAGACGGCATACGAGAT ACGGAACT GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	AGTTCCGT
Index L03	CAAGCAGAAGACGGCATACGAGAT TCTGACAT GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	ATGTCAGA
Index L04	CAAGCAGAAGACGGCATACGAGAT CGGGACGG GTGAC TGGAGTTCAGACGTGTGCTCTTCCGATC*T	CCGTCCCG
Index L05	CAAGCAGAAGACGGCATACGAGAT GTGCGGAC GTGAC TGGAGTTCAGACGTGTGCTCTTCCGATC*T	GTCCGCAC
Index L06	CAAGCAGAAGACGGCATACGAGAT CGTTTCAC GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	GTGAAACG
Index L07	CAAGCAGAAGACGGCATACGAGAT AAGGCCAC GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	GTGGCCTT
Index L08	CAAGCAGAAGACGGCATACGAGAT TCCGAAAC GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	GTTTCGGA
Index L09	CAAGCAGAAGACGGCATACGAGAT TACGTACG GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	CGTACGTA
Index L10	CAAGCAGAAGACGGCATACGAGAT ATCCACTC GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	GAGTGGAT
Index L11	CAAGCAGAAGACGGCATACGAGAT ATATCAGT GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	ACTGATAT
Index L12	CAAGCAGAAGACGGCATACGAGAT AAAGGAAT GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC*T	ATTCCTTT

The full length of index primer is 66 bp
* indicates phosphorothioate bond, *Underlined* is index sequence, # expected index primer sequencing read

on the gel by combining the two LR PCR reactions that were set up for each sample into a single 1.5 mL LoBind® tube.

5. Aliquot 0.7× volume of the Ampure XP beads (*see* **Note 6**) into a new LoBind® tube. For example, if sample volume is 100 μL, then 70 μL beads should be added. Transfer the LR-PCR product into LoBind® tube, combining products from two duplicated set-up for each sample, total is about 96 μL. Vortex for 2 s.
6. Let the tubes sit at room temperature (RT) for 5 min.
7. Briefly spin down the tubes and put them on the magnetic stand. Wait for 3 min until the solution becomes clear.

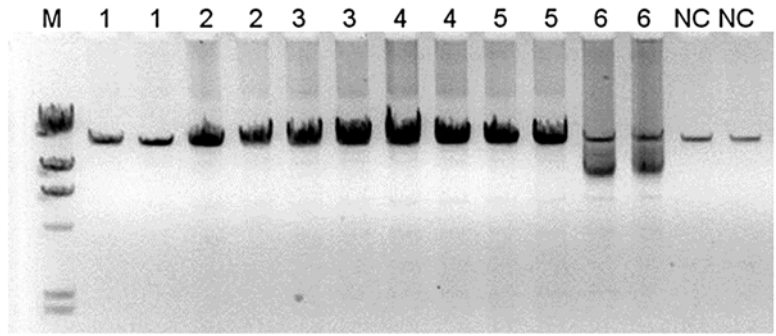

Fig. 1 Long-range PCR gel picture. *M* λ DNA-HindIII Digest marker, *NC* negative control, 1–6: patient samples, run in duplicate. Sample 6 has a heteroplasmic mtDNA large deletion

8. Pipet out the supernatant without touching the beads. Discard the supernatant.
9. Do not remove the tubes from the stand. Add 500 μL of 70 % EtOH. Let the tubes containing the beads, PCR products and the 70 % EtOH stand for 1 min on the magnetic stand, then pipet the supernatant out. Discard the supernatant. Repeat this step once.
10. Put the tubes in a 37 ± 0.5 °C heat block for 5 min or until EtOH is fully evaporated.
11. Elute the beads, which contain the purified PCR DNA products, with 51 μL ddH_2O.
12. Vortex the tubes and let them sit at room temperature (RT) for 5 min.
13. Put the tubes back on the magnetic stand for 2 min until solution is clear.
14. Transfer 50 μL of the supernatant (containing the purified and eluted PCR products) from each tube directly into PCR strip tubes and close the caps after each transfer.
15. For each sample to be quantified, prepare working solution by mixing 199 μL of Qubit High Sensitivity (HS) buffer with 1 μL of HS dye for each reaction (*see* **Note 7**).
16. Make sure to prepare sufficient working solution for the samples to be quantified, plus two different standards (standard 1 is 0 ng/μL and standard 2 is 10 ng/μL, the quantification range is 0.2–100 ng). For example, if there are 12 samples, prepare working solution for 12 samples + 2 standards + 1 extra = 15 total. Total working solution equals 200 μL × 15 = 3 mL.
17. To each Qubit assay tube add 199 μL of the working solution and 1 μL of the DNA sample. For each standard, add 190 μL

of the working solution to a Qubit assay tube and 10 μL of the standard. The average concentration of the LR PCR product is 70 ng/μL. 1500 ng of a sample with a concentration of 70 ng/μL would be 21.43 μL of the LR product (1500/70 = 21.43).

3.2 PCR Product Fragmentation

1. Add a total of 1500 ng of purified LR-PCR product to a DNA LoBind tube. If there is insufficient amount of LR-PCR product, as little as 750 ng may work.
2. Bring the final volume to 125 μL with ddH_2O for fragmentation.
3. Turn on the cooling system. The bath temperature needs to be at 4 ± 2 °C (*see* **Note 8**).
4. Open the method labeled 175 bp that corresponds to the plate being used. The settings will show up on the screen as such: Treatment Time (s) 360; Acoustic Duty Factor (%) 10; Peak Incident Power (W) 175; Cycles per Burst 200.
5. Transfer the 125 μL sample mixture in each LoBind® tube to a labeled microTUBE. Remove any bubbles by gently tapping the tube bottom. Carefully place the microTUBEs on the Covaris plate. Edit the method to reflect which wells need to be fragmented on the plate and save the method.
6. Insert the plate into the water bath and close the door. Click start.
7. After the program is finished (~6 min per sample), transfer the sample from microTUBEs into 1.5 mL DNA LoBind® tubes. Store samples on ice.

3.3 Purification of the Fragmented PCR Product

1. Purify the fragmented product using Ampure XP beads as described (Subheading 3.1, **steps 4–14**) with the following changes:
2. In **step 5**, aliquot 180 μL beads.
3. In **step 11**, elute the dried beads with 17 μL (ddH_2O).
4. In **step 14**, transfer 15 μL of the purified product from each sample tube directly into PCR strip tubes and close the caps after each transfer.

3.4 End Repair and Purification

1. Mix the following components in a sterile microfuge tube at final volume of 50 μL per sample:

   5 μL of 10× NEBNext End Repair Reaction Buffer, final 1×.

   2.5 μL of NEBNext End Repair Enzyme Mix.

   15 μL of purified, fragmented DNA from Subheading 3.3, containing a total of about 1500 ng DNA.

   Add ~27.5 μL of ddH_2O to a final volume of 50 μL.
2. Incubate in a thermal cycler at 20 °C for 30 min.

3. Purify the fragmented product using Ampure XP beads as described (Subheading 3.1, **steps 4–14**) with the following changes:
4. In **step 5**, aliquot 90 μL beads.
5. In **step 11**, elute the dried beads with 23 μL (ddH_2O).
6. In **step 14**, transfer 21 μL of the purified product from each sample tube directly into PCR strip tubes and close the caps after each transfer.

3.5 Adenylation

1. Each adenylation reaction at a final volume of 25 μL, requires the following components: 2.5 μL of 10× NEBNext dA-tailing Reaction Buffer and 1.5 μL of Klenow fragment (3′ to 5′ exo$^-$).
2. Amount depends on the number of samples to be adenylated, mix sufficient amount of these two components in a master mix. For example, if there are ten samples to be processed, mix 25 μL of 10× NEBNext dA-tailing Reaction Buffer and 15 μL of Klenow fragment, to a total of 40 μL master mix. Calculate total master mix volume based on sample number.
3. Add 4 μL of above master mix to each end repaired sample tube (from Subheading 3.4). Close cap after each template is added.
4. Incubate in a thermal cycler at 37 °C for 30 min.
5. Purify the fragmented product using Ampure XP beads as described (Subheading 3.1, **steps 4–14**) with the following changes:
6. In **step 5**, aliquot 45 μL beads.
7. In **step 11**, elute the dried beads with 16 μL (ddH_2O).
8. In **step 14**, transfer 13.5 μL of the purified product for each sample directly into PCR strip tubes and close the caps after each transfer.

3.6 Adapter Annealing

1. Mix the following components in a sterile microfuge tube to a final volume of 100 μL:

   10 μL of 10× PCR buffer (without $MgCl_2$), final 1×.

   30 μL of 100 μM Multiplexing Adapter Forward, final 30 μM.

   30 μL of 100 μM Multiplexing Adapter Reverse, final 30 μM.

   30 μL of ddH_2O to a final volume of 100 μL.
2. PCR cycling:

Lid:	105 °C, Wait, Auto
Block:	95 °C, 10 min
	60 °C, 5 min × 35 cycle (−1 °C each cycle)
	4 °C, hold

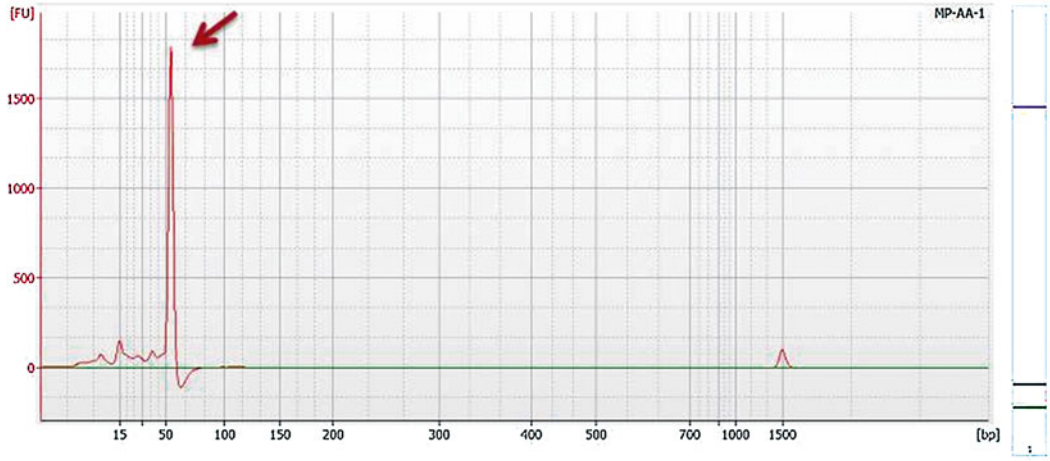

Fig. 2 BioAnalyzer result for synthesized adaptor. *Arrow* indicates the adaptor peak at size of 53 bp

3. Use 1 μL of each sample to check on BioAnalyzer using a DNA 1000 Chip. Use known working adapter as a control (15 μM is optimal) (Fig. 2).
4. Turn on the 2100 BioAnalyzer.
5. Open the Agilent 2100 Expert Software and check communication.
6. Prepare the chip, samples, and ladder as instructed in the reagent kit guide.
7. Load the prepared chip into the 2100 BioAnalyzer and start the run within 5 min after preparation.
8. Within the instrument context, choose the DNA 1000 assay from the drop-down list.
9. Enter sample names and comments in the Data and Assay context.
10. Start the run. It will take 45 min to finish 12 samples.
11. Quantify with regions.
12. Verify and save the results.
13. Combine up to six completed adapter anneal reactions (adapters) into a 1.5 mL tube, label with concentration, and store at −20 °C. 100 μL of adapter at approximately 15 μM is enough for 25 samples. The adapters will last up to a year when stored at −20 °C, so multiple reactions can be set up.

3.7 Ligation and Purification

1. Each 25 μL ligation reaction contains the following components:

   5 μL of 5× Quick Ligation Reaction Buffer, final 1×.

   4 μL of the 15 μM annealed Index PE adapter mix (from Subheading 3.6), final 2.4 μM.

2.5 μL of Quick T4 DNA ligase.

13.5 μL of A-tailed sample DNA (from Subheading 3.5).

2. Prepare the master mix without the A-tailed DNA template first by mixing the first three components sufficient for the number of samples to be processed. Then, pipet 11.5 μL of the master mix to the PCR strip tubes that already contain 13.5 μL of A-tailed DNA template from Subheading 3.5. Close cap after each template is added.
3. Incubate in a thermal cycler for 15 min at 20 °C.
4. Purify the fragmented product using Ampure XP beads as described (Subheading 3.1, **steps 4–14**) with the following changes:
5. In **step 5**, aliquot 45 μL beads.
6. In **step 11**, elute the dried beads with 17 μL (ddH_2O).
7. In **step 14**, transfer 15 μL of the purified product for each sample into 1.5 mL tubes, label, and store at −20 °C.
8. Check the Adapter Ligated product on the BioAnalyzer using a DNA 1000 chip (Subheading 3.6, **steps 4–12**).
9. There will be a 53 bp addition after the adapter is ligated. You should see the peaks shift to the right-hand side accordingly (Fig. 3).

3.8 PCR Enrichment of DNA template

1. Each 50 μL PCR enrichment reaction contains the following components:

1 μL of 25 μM InPE 1.0 (F), final 0.5 μM.

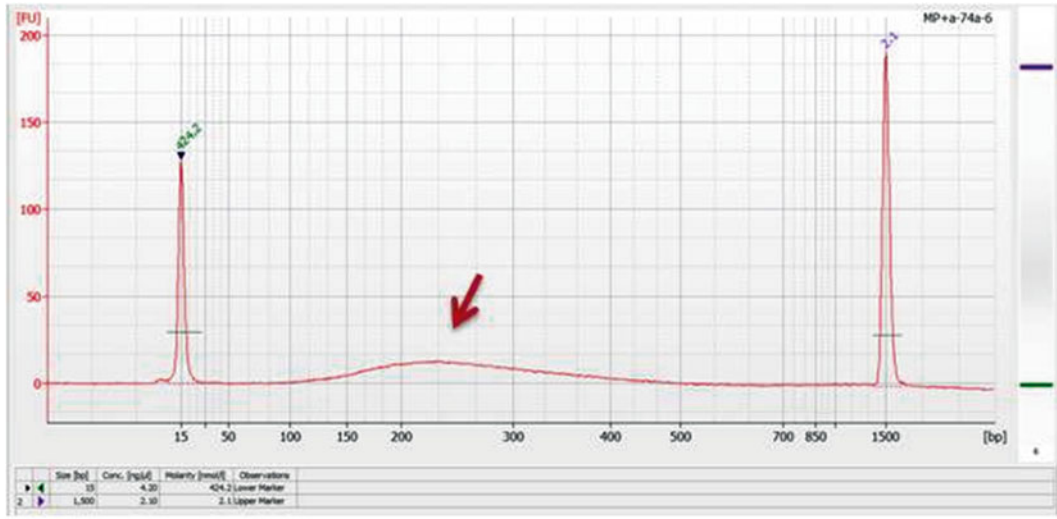

Fig. 3 BioAnalyzer result for ligation products. The two peaks are low and high molecular markers of 15 and 1500 bp, respectively. *Arrow* indicates the ligation products at peak around 208 bp

10 μL of 5× Herculase II reaction buffer, final 1×.

0.5 μL of 25 mM dNTP, final 0.25 mM.

1 μL of Herculase II polymerase.

Add 32.5 μL of ddH_2O to a final volume of 45 μL.

2. In each of 45 μL master mix, add 1 μL of the indexing primer (final 0.5 μM, Table 1) and 4 μL of adapter ligated product (from Subheading 3.7).
3. Prepare sufficient amount of master mix depending on the number of samples to be processed.
4. Using PCR strip tubes, add 45 μL master mix to each tube first, then add 1 μL of the indexing primer (Table 1) and 4 μL of adapter ligated product (from Subheading 3.7). Close cap after each template is added.
5. PCR cycling:

Lid:	105 °C, Wait, Auto	
Block:	98 °C, 30 s	
	98 °C, 10 s 60 °C, 30 s	× 8 cycles[a]
	72 °C, 30 s	
	72 °C, 5 min	
	4 °C, hold	

[a]Can run 6–10 cycles depending on the concentration.

6. Purify the fragmented product using Ampure XP beads as described in Subheading 3.1 with the following changes:
7. In **step 5**, aliquot 90 μL beads.
8. In **step 11**, elute the dried beads with 33 μL ddH_2O.
9. In **step 14**, transfer 30 μL of the purified product for each sample into 1.5 mL tubes, label, and store at −20 °C.
10. Check the enriched product on the BioAnalyzer using a DNA 1000 Chip (Subheading 3.6, **steps 4–12**).
11. There will be an additional 71 bp in size after the product is enriched. The peaks shift to the right-hand side accordingly.
12. Check the concentration of purified final products using Qubit HS assay (*see* **Note 7**).

3.9 Pooling Libraries and Sequencing

1. Combine the DNA template libraries (from Subheading 3.8) with different indexes in equal molar amount. Use the average concentration of the BioAnalyzer result and the Qubit result. Each pooled library will have a concentration of 10 nM (*see* **Note 9**).

2. Save the pooled samples at −20 ± 2 °C. DNA template library preparation is completed. Samples will be handed over for cluster generation and sequencing.
3. Quantify the Final library Pools by qPCR. Dilute each library pool to a concentration between 20 and 0.0002 picomolar (pM) with a 10^3, 10^4, and 10^5 dilution (First transfer 1 μL undiluted library to 999 μL ddH_2O for the 10^3 dilution, vortex well, then transfer 10 μL of the 10^3 dilution to 90 μL ddH_2O for 10^4 dilution, and transfer 1 μL of the 10^3 dilution to 99 μL ddH_2O for 10^5 dilution).
4. Add 1 mL of Illumina GA Primer Premix (10×) to 5 mL bottle of KAPA SYBR FAST qPCR Master Mix (2×) and mix well. This is the KAPA SYBR FAST qPCR Master Mix containing Primer Premix (see below). Do this step only with a new KAPA kit.
5. Each 10 μL 1× reaction contains the following components:

   6 μL of KAPA SYBR FAST qPCR Master Mix containing Primer Premix.

   2 μL ddH_2O.

   2 μL Diluted library DNA or DNA standard.
6. Each sample and standard should be assayed in duplicate.
7. Place the reactions in the real-time thermocycler, and set program as shown below:

Initial activation/denaturation:	95 °C, 3 min	
Denaturation: Annealing/extension/data acquisition:	95 °C,10 s 60 °C, 45 s	×40 cycles
Add dissociation step		

8. Calculate and record each final library concentration.
9. Sequence using the Illumina HiSeq 2000 and follow the protocol provided by Illumina.

3.10 Results and Data Analysis

1. Convert raw data in base call files (.bcl format) to qseq files before demultiplexing with CASAVA v1.7 software (Illumina Inc., San Diego, CA, USA).
2. Demultiplexed data are processed further by NextGENe software for alignment (SoftGenetics, State College, PA, USA) (Fig. 4).

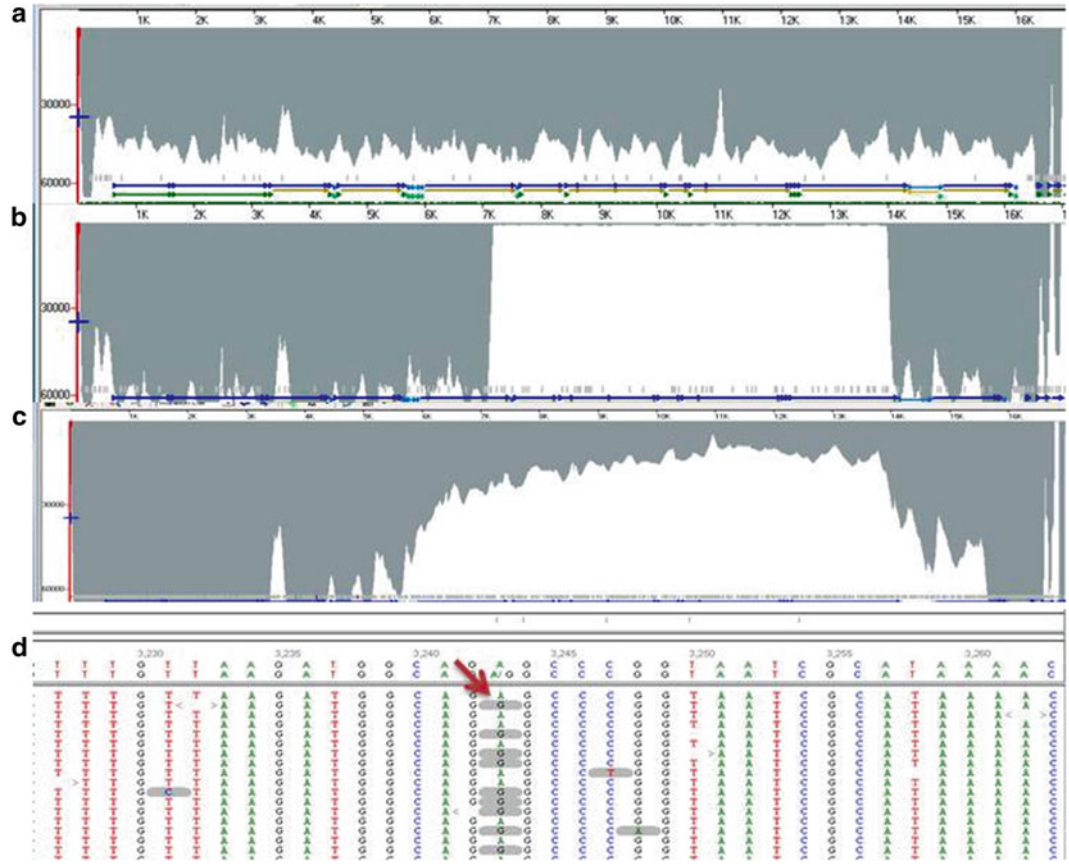

Fig. 4 Examples of massively parallel sequencing results for mitochondrial genome. (**a**) Even coverage for whole mitochondrial genome at average depth >30,000×. (**b**) Absent coverage between m.7,000 and m.14,000 indicates a large mtDNA deletion of about 7 Kb. (**c**) Curved mitochondrial coverage profile indicates mtDNA multiple deletions in a muscle specimen. (**d**) Detection of a heteroplasmic m.4343A > G mutation

4 Notes

1. Primers used for LR PCR to amplify whole mitochondrial genome. Amplicon size is 16.6 kb.

   Forward primer (mt16426F) 5′-ccgca caagagtgct actctcctc-3′

   Reverse primer (mt16425R) 5′-gatattgatttcacggaggatggtg-3′

2. DNA can be extracted from blood, cultured cells or freshly frozen tissue, and dissolved in DNA hydration buffer. Store at 4 °C.

3. Primer Sequences for multiplex adapter:

   Forward primer: 5′-ACACTCTTTCCCTACACGACGCTCT TCCGATC*T -3′.

   Reverse primer: 5′-P-GATCGGAAGAGCACACGTCT-3′.

Make 100 μM stock of each with ddH_2O or elusion buffer.

***** indicates phosphorothioate bond.

4. Primer Sequences for IndexPE1.0 (F):

   5′-AATGATACGGCGACCACCGAGATCTACA CTCTTTCCCTACACGACGCTCTTCCGATCT-3′.

5. DNA Template: Dilute DNA to 50 ng/μL for blood and fibroblast specimens and 10 ng/μL for muscle and liver specimens, add 2 μL DNA template in a 50 μL reaction mix. Set up the reactions on a PCR cool plate.
6. AMPure XP beads (stored at 4 ± 2 °C) need to come to room temperature before use. Invert the tube containing the beads several times to make the solution homogenous. Prepare fresh 50 mL 70 % EtOH before starting by adding 35 mL 100 % EtOH to 15 mL ddH_2O. The solution can be stored at room temperature, and can be used for future purification steps for up to a week.
7. Qubit standards (stored at 4 ± 2 °C) need to come to room temperature before use. Take it out 30 min before the assay. Follow instructions on the Qubit fluorometer to determine each sample's concentration. If DNA concentration is off the curve, or too high, make a 10× dilution of the sample and repeat the assay.
8. The water bath temperature needs to be at 4 ± 2 °C before fragmentation starts. Turn on at least 30 min ahead of time and make sure that it has cooled down before use. Fill the tank with milliQ water up to the fill level on the water tank. Turn on Covaris, open the Covaris program, and start degassing. Degassing needs to be done for at least 45 min. The E-Series has a conditioning system that keeps the water pure. This enables the machine to be left on indefinitely.
9. For each library, use the formula below to determine the amount of index sample to use:

$$\text{Volumeofindex}(\mu\text{L}) = \frac{vf\,(\mu\text{l}) \times 10\text{nM}}{12 \times Ci}$$

Vf final desired volume of the pool, *Ci* concentration (nM) of individual library before pooling.

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Chapter 2

Analysis of Translesion DNA Synthesis by the Mitochondrial DNA Polymerase γ

William C. Copeland, Rajesh Kasiviswanathan, and Matthew J. Longley

Abstract

Mitochondrial DNA is replicated by the nuclear-encoded DNA polymerase γ (pol γ) which is composed of a single 140 kDa catalytic subunit and a dimeric 55 kDa accessory subunit. Mitochondrial DNA is vulnerable to various forms of damage, including several types of oxidative lesions, UV-induced photoproducts, chemical adducts from environmental sources, as well as alkylation and inter-strand cross-links from chemotherapy agents. Although many of these lesions block DNA replication, pol γ can bypass some lesions by nucleotide incorporation opposite a template lesion and further extension of the DNA primer past the lesion. This process of translesion synthesis (TLS) by pol γ can occur in either an error-free or an error-prone manner. Assessment of TLS requires extensive analysis of oligonucleotide substrates and replication products by denaturing polyacrylamide sequencing gels. This chapter presents protocols for the analysis of translesion DNA synthesis.

Key words DNA polymerase γ, Mitochondrial DNA polymerase, DNA replication, Translesion synthesis, DNA repair, Enzyme assays, *POLG*

1 Introduction

The mitochondrial (mt) genome is a multicopy closed circular genome of 16,569 bp that encodes 13 proteins involved in the electron transport chain, 22 transfer RNA genes, and 2 ribosomal RNAs required for mitochondrial protein synthesis of the 13 polypeptides. Cells contain several thousand copies of mtDNA distributed within hundreds of mitochondria. The mtDNA is located in the mitochondrial matrix within discrete nucleoids, each containing 1–2 copies of mtDNA [1].

Mitochondrial DNA incurs chemical damage from both endogenous and exogenous sources, which can result in mutations [2]. Leakage of electrons from the electron transport chain generates reactive oxygen species, which are the major source of oxidative damage to mtDNA. Mitochondria are able to repair oxidative

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lesions because they possess a robust base excision repair (BER) system, but the lack of an efficient mismatch repair system and the marked absence of nucleotide excision repair result in the persistence of miscoding lesions in mtDNA [3]. Because unrepaired lesions can promote mtDNA mutations or even block DNA replication, the ability of pol γ to bypass such lesions by TLS is vital to maintaining the genetic integrity of mtDNA. In contrast to the nucleus, which possesses several specialized DNA polymerases to efficiently bypass different DNA lesions, mammalian mitochondria appears to contain a single DNA polymerase, pol γ, which bears the burden both to replicate the 16.5 kb circular mitochondrial genome and to participate in DNA repair [4–13]. The human DNA pol γ holoenzyme is a heterotrimeric complex comprised of a catalytic subunit, encoded by *POLG* at chromosomal locus 15q25, and a homodimeric accessory subunit, encoded by *POLG2* at chromosomal locus 17q24.1. The catalytic subunit, a member of the A family of DNA polymerases, is a 140 kDa enzyme (p140) that possesses DNA polymerase, $3' \rightarrow 5'$ exonuclease and 5′-dRP lyase activities (reviewed in [13]). The accessory subunit, a homodimer of two 55 kDa proteins (two p55 monomers) binds asymmetrically to the catalytic subunit, where the proximal p55 enables tight DNA binding and the distal monomer confers processive DNA synthesis to the holoenzyme [14, 15].

Translesion DNA synthesis by the human DNA pol γ has been studied for a number of DNA lesions, including 7,8-dihydro-8-oxo-2′-deoxyguanosine [16], benzopyrene adducts [17], UV photoadducts [18], acrolein adducts [19], and several others that are reviewed in [2]. We evaluate translesion DNA synthesis by pol γ with synthetic oligonucleotide substrates that contain specific lesions of interest. DNA polymerization reactions are utilized to measure the efficiency of pol γ to incorporate opposite and to extend beyond specific DNA lesions in vitro. This paper describes the general procedures needed to measure translesion DNA synthesis and efficiency of lesion bypass with the purified DNA polymerase γ.

2 Materials

2.1 Enzymes

1. Recombinant catalytic subunit of human DNA pol γ (exonuclease-proficient and exonuclease-deficient forms) containing a His_6 affinity-tag at its N-terminus was overproduced in baculovirus-infected Sf9 cells, and the protein was purified to homogeneity as described previously [20–22].
2. The p55 accessory subunit containing a His_6 affinity-tag at its C-terminus was expressed in *E. coli* and purified to homogeneity as described [20–23].

2.2 Primer Template Substrates

1. Primer template substrates with and without damage were designed and synthesized. A control oligonucleotide primer template pair with no damage is designed, constructed, and purchased from Integrated DNA Technologies. An identical template with a site-specific lesion replacing a normal nucleotide is also synthesized. As an example, investigation of translesion synthesis opposite *uv*-photodimers utilized the following undamaged DNA template [18]:

5′-AATTTCTGCAGGTCGACTCCAAAGGCT-3′	Primer
3′-TTAAAGACGTCCAGCTGAGGTTTCCGA**TT** GGGCCATGGCTCGACC-5′	Template

   For TLS synthesis, the template was redesigned with a cycloprimidine dimer at positions 17 and 18 from the 5′ end [18], depicted above in **bold**.

2. Our investigation into translesion synthesis opposite an acrolein adduct (γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-HOPdG)) utilized the following primer template pair [19]:

5′-GGG GGC TCG TAA GGA TTC-3′	Primer
3′-CCC CCG AGC ATT CCT AAG (γ-HOPdG) CT GA-5′	Template

   The corresponding oligodeoxynucleotide template with undamaged dG at position 5 was purchased from Integrated DNA Technologies:

   3′-CCC CCG AGC ATT CCT AAG **G**CT GA-5′

2.3 Primer Extension Assays

1. Enzyme dilution buffer: 50 mM Tris–HCl, pH 7.5, 10 % glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 50 μg/mL acetylated bovine serum albumin (BSA), 0.1 M NaCl.
2. T4 polynucleotide kinase.
3. Primer template extension buffer: 25 mM HEPES–KOH, pH 7.5, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM $MgCl_2$.
4. Phosphor storage screen.
5. Typhoon 9400 phosphorimager.
6. NIH Image or ImageJ software (http://rsb.info.nih.gov/ij/).
7. Non-linear kinetic curves are generated using KaleidaGraph software, Version 4.1.3 (Synergy Software).

2.4 Sequencing Gel Electrophoresis

1. Model S2 Sequencing Gel Electrophoresis apparatus (Life Technologies) or equivalent.
2. 40 % acrylamide/Bis solution, 19:1.

3. 10× Tris–Borate EDTA (TBE) solution: 108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA, pH 8.0 in 1 L deionized H_2O.
4. Ultrapure Urea.
5. TEMED (*N*,*N*,*N*′,*N*′-tetra-methyl-ethylenediamine).
6. 10 % ammonium persulfate solution: 1 g ammonium persulfate dissolved in 10 mL dH_2O.
7. 0.4 mm × 50 well flat-tooth comb.

3 Methods

3.1 5′-End Labeling of the Primer

1. In a chilled 1.5 mL polypropylene microfuge tube, add 10× T4 polynucleotide kinase buffer (to produce a final concentration of 1×), 20 pmol of the gel-purified primer, 25 pmol [γ-^{32}P]ATP, 12 U T4 polynucleotide kinase and dH_2O to 25 μL.
2. Incubate reactions at 37 °C for 60 min.
3. Heat at 95 °C for 5 min to inactivate the kinase.
4. Cool tube on ice.

3.2 Hybridization of Primer Template

1. Mix 10 pmol ^{32}P-labeled primer with 12 pmol template oligonucleotide. Add TE buffer to 100 μL.
2. Vortex gently. Heat tube in a 400 mL beaker containing 95 °C H_2O for 5 min.
3. Cool slowly to room temperature by leaving the beaker at room temperature.
4. Store primer template at –20 °C until needed.

3.3 Primer Template Extension Reactions

1. Dilute and premix the polymerase subunits (gently mixing p140 and p55 in a 1:2 molar ratio) in enzyme dilution buffer to desired concentration. Premixed enzymes are added last to assembled reaction mixtures (10 μL) on ice (*see* **Notes 1** and **2**).
2. Combine 50 nM radiolabeled primer template substrate, 10 nM exonuclease-deficient p140 and 20 nM p55 in 10 μL of primer template extension buffer. Reactions are initiated by the addition of one of the four dNTPs (at different concentrations depending on the substrate and analysis). Reactions lacking p55 accessory subunit are performed with no added NaCl, whereas reactions containing the p55 accessory subunit are supplemented to a final concentration of 100 mM NaCl.
3. Reactions are incubated at 37 °C for 2–10 min, depending on the desired extent of incorporation.
4. Reactions are stopped by the addition (10 μL) of 95 % deionized formamide and 10 mM EDTA and stored at –20 °C prior to further analysis.

3.4 Separation of Reaction Products on a Sequencing Gel

1. Prepare a polyacrylamide sequencing gel containing 8 M urea by dissolving 33.6 g urea with 28 mL of 40 % acrylamide/Bis solution (19:1), 7 mL of 10× TBE and dH_2O to a final volume of 70 mL. Warm slightly to dissolve urea.
2. Filter solution through Whatman paper in a Buchner funnel to remove impurities and degas for 15 min while sitting in a chilled solution of water (*see* **Note 3**). Add 630 μL 10 % APS and 15 μL of TEMED, mix gently and quickly pipet solution between sequencing gel plates with 0.4 mm spacers. Insert a 0.4 mm × 50 well flat-tooth comb (not a sharks tooth comb) to form the wells. Allow gel to polymerize for 1 h.
3. Remove the comb and clamp the gel in the Model S2 Sequencing Gel apparatus. Fill chambers with 1× TBE buffer and pre-run gel for 45 min at 70 W.
4. Heat samples at >95 °C for 5 min, load samples (3 μL per well), and resolve the products by electrophoresis through the denaturing 16 % polyacrylamide gel.
5. Dry the gel and expose to a phosphor storage screen (*see* **Note 4**).
6. Image the radioactivity on a Typhoon 9400 phosphorimager (Fig. 1).

3.5 Calculations

1. Quantify bands using NIH ImageJ software.
2. Kinetic constants are determined by integration of the band intensities (*see* Fig. 1). Velocity is determined from the ratio of the band intensities using the NIH Image J software as described [24, 25] where I_0 is the intensity of the primer band and I_1 is the intensity of the primer +1 incorporation product. Reaction velocity is $v = I_1/(I_0 + I_1)$ [24, 25]. This is termed the standing start reaction (*see* **Note 5**).

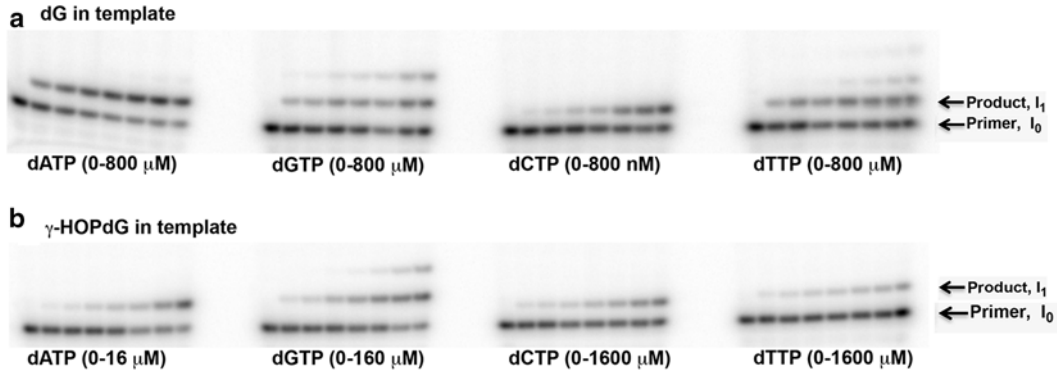

Fig. 1 Representative single nucleotide incorporation reactions catalyzed by the exonuclease-deficient form of DNA pol γ opposite a control primer template with a normal dG (**a**) and the γ-HOPdG lesion in the DNA template (**b**) [19]. Reactions were performed with increasing concentrations of dATP, dGTP, dCTP, and dTTP, as indicated

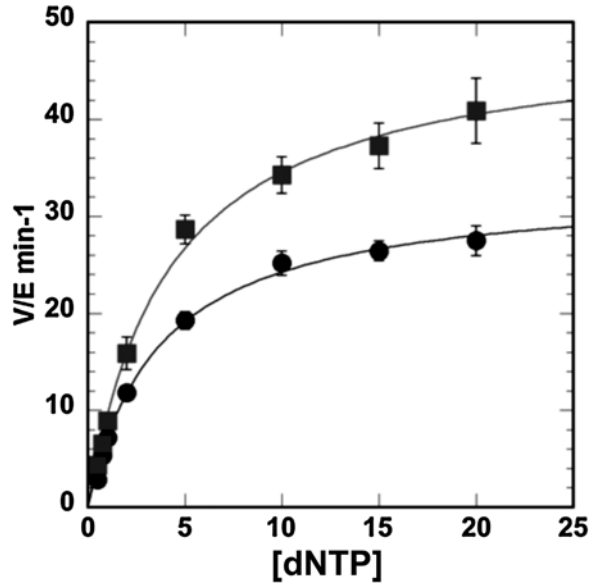

Fig. 2 Representative steady-state kinetics of pol γ. Steady-state incorporation of dATP (*squares*) and dGTP (*circles*) opposite (γ-HOPdG) [19], as fit to the Michaelis–Menten equation. The k_{cat} values (V/E min^{-1}) were plotted versus μM amounts of dATP or dGTP

3. The steady-state kinetic parameters K_m and V_{max} were determined by fitting the data to the Michaelis–Menten model using KaleidaGraph (Version 4.1.3, Synergy) (*see* Fig. 2). The relative efficiency of incorporation opposite a specific DNA adduct compared to incorporation opposite a normal template nucleotide is estimated as the ratio of the kinetic constants for adducted DNA divided by the ratio for undamaged DNA ($f_{in} = (\text{kcat}/K_m(\text{adduct}))/(\text{kcat}/K_m(\text{normal}))$.

4 Notes

1. Assemble all reactions in an ice bucket. Keeping the enzymes cold is essential, as pol γ has a functional half-life at 42 °C of less than 2 min in the absence of the accessory subunit and DNA [26].
2. Refreeze enzyme stock tubes as soon as possible in liquid nitrogen and store at −80 °C. Enzyme preparations begin to lose DNA polymerase activity after approximately three freeze–thaw cycles. The enzyme buffer contains glycerol, salt and 2-mercaptoethanol, which are very important for stability.
3. Degassing is needed to remove oxygen that will slow the polymerization reaction while chilling on ice is needed to slow down the polymerization once TEMED and APS are added.

This provides sufficient time to pour the gel between the plates prior to polymerization.

4. Transferring the polyacrylamide gel to Whatman paper for drying is difficult and can result in tearing of the gel if done improperly. Ensure that the smaller glass plate is siliconized, which will easily allow the gel to detach while still sticking to the larger plate. The gel can then be transferred from the glass to plastic wrap, where any wrinkles can be flattened out, and finally sandwiched with a sheet of Whatman paper.
5. Running start reactions can also be performed with a primer terminus recessed three nucleotides proximal to the position of the lesion in the template. In this case, velocity is determined from the ratio of I_3/I_2 [24, 25]. Similarly, extension past a lesion can also be assessed using a primer extended by one nucleotide where the primer's 3′-end is positioned opposite the lesion.

Acknowledgments

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Chapter 3

Quantification of DNA Damage by Real-Time qPCR

Wei Wang, Katja Scheffler, Ying Esbensen, and Lars Eide

Abstract

This chapter describes the use of real-time qPCR to quantify damages in genomic DNA. The method is based on the ability of a lesion in one strand to inhibit restriction enzyme digestion of double-stranded DNA. Subsequent amplification of the complementary strand after restriction cleavage gives a quantitative measure of the damage content in that site (*R*eal-time qPCR *A*nalysis of *D*amage *F*requency; RADF). We compare the RADF assay with the commonly used technique to assess damages by their ability to inhibit amplification of a large PCR fragment relative to a short PCR fragment. The RADF method described here is quick, accurate and allows the detection of nuclear and mitochondrial DNA damage in detailed regions.

Key words DNA damage, mtDNA, DNA repair

1 Introduction

Nuclear and mitochondrial genomes are continuously exposed to reactive oxygen species (ROS) derived from numerous endogenous and exogenous sources that induce oxidative damage to DNA. In aerobic organisms, direct reduction of oxygen can occur in the electron transport chain process in mitochondria but also in peroxisomes during the degradation of long fatty acids [1, 2]. Oxygen derivatives include hydroxyl radical, superoxide anion, and hydrogen peroxide, with increasing stability as well as ability to cross membranes. Hence, cytosolic ROS production can readily attack nuclear as well as mitochondrial DNA (mtDNA). Increased level of ROS, as in oxidative stress, or alternatively under defective or inefficient repair trigger accumulation of oxidative DNA damage. DNA damage has been implicated in various human diseases including cancer, neurodegenerative disorders, and the aging process [3]. ROS-induced modifications of DNA include base lesions, modification on the ribose, and single- and double-strand breaks [4]. Estimation of DNA damage is not only relevant for these topics, but also as an indirect tool to predict intracellular oxidative stress.

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Precise quantification of DNA damage is challenging, and the resolution of the quantitative assay occurs on the cost of specificity of the damage and amount of material required. Direct detection of damage as in antibody detection of a specific base lesion [5], or electrochemical or mass spectrometry detection of a lesion separated on a HPLC is hampered by variability of the antibody quality and low signal/noise ratio [6]. Indirect methods to identify DNA modifications include DNA glycosylase incision of a DNA-containing base lesions, followed by denaturing electrophoresis (comet-like type) [7] or effective amplification of very long amplicons in a quantitative PCR [8]. The latter has been widely used to assess mtDNA damage, partly because the multicopy presence of mtDNA allows analysis on limited amount of material. The method is based on the assumption that damage accumulate in a Poisson distribution, and quantification is achieved by transformation of the Poisson distribution equation to yield the lambda value: the expected amount of amplicons that are devoid of damage. Recent advances have shown that this principle is workable in a real-time qPCR amplifying a shorter product. The disadvantage of this method including the other abovementioned methods is that the region specificity is low. This is of matter when the aim is to compare regions within a gene, e.g., promoter versus intergenic damage, or alternatively introns versus exons. The requirement for long PCR fragment is also challenging with several commercially available DNA extraction kits as they to some degree lead to DNA fragmentation.

Here, we describe a qPCR-based method for DNA damage quantification that is based on the ability of a base lesion (or possible other DNA modifications) to inhibit restriction enzyme cleavage. Upon treatment with a restriction enzyme, double-stranded DNA will be digested unless this is prevented by damage. Subsequent amplification of the complementary, damage-free DNA strand in a real-time quantitative qPCR allows quantitative assessment of DNA damage with high site specificity. We provide primer sets that have been used to quantify DNA damage in mouse mtDNA and nDNA. However, the assay can be adjusted to detect damage in any site containing a restriction enzyme recognition sequence with primers spanning this site. Because the amplicon to be amplified is in the order of hundred base pairs, the method is not sensitive to fragmentation from isolation.

2 Materials

1. NanoDrop spectrophotometer.
2. 7900HT Fast Real-Time PCR System.
3. DNA isolation kit: DNeasy Blood and Tissue kit.
4. 100 % Ethanol.

Table 1
Oligonucleotides used for DNA damage quantification

Target	Forward	Reverse
mt-Rnr1	actcaaaggacttggcggta	agcccatttcttcccatttc
Ndufa9	tggtgactcctacctgaagc	ttcggtcgtgaattttgttt
117 bp mtDNA fragment	cccagctactaccatcattcaagt	gatggtttgggagattggttgatgt
10 kb mtDNA fragment	gccagcctgacccatagccataatat	gagagattttatgggtgtaatgcgg
8-oxoG containing duplex (G replaces 8oxoG in control forward oligonucleotide)	tggttgacctgggcgtaaaatctagatc **8oxog**aaacgttacaaaggtgagaa gatcggtcag	ctgaccgatcttctcacctttgtaacg tttcgatctagattttacgcccagg tcaacca

5. Nuclease-free water.
6. Power SYBR Green PCR mixture.
7. TaqI Restriction Enzyme.
8. Primers used for analyzes of nuclear and mtDNA is provided in Table 1.
9. Hydrogen peroxide (H_2O_2).
10. CL-1000 Ultraviolet Crosslinker.
11. Methyl methanesulfonate (MMS).
12. Dulbecco's Modified Eagle Medium (DMEM).

3 Methods

3.1 Extraction of Total DNA

Total DNA is isolated from cultured cells or tissues using the DNeasy Blood and tissue kit according to the manufacturer's protocols. The DNA is quantified by NanoDrop analyses (*see* **Note 1**).

1. Based on NanoDrop quantification, adjust template DNA concentration to 2 ng/μL for mtDNA or 10 ng/μL for nDNA analysis.

3.2 TaqI Digestion and Real-Time qPCR Analysis

2. Prepare master qPCR reaction mixture with or without 1 unit/well of TaqI restriction enzyme. The qPCR reaction mixture without TaqI serves as non-digestion control which indicates amounts of DNA molecules loaded.
3. 6 ng or 30 ng (3 μL) of total DNA is added to both non-TaqI and TaqI containing qPCR reaction mixture.
4. qPCR is performed using selected primers in a 7900HT Fast Real-Time PCR System with the Power SYBR® green PCR Master mixture.
5. A step of 65 °C for 15 min is included prior to the standard qPCR program.

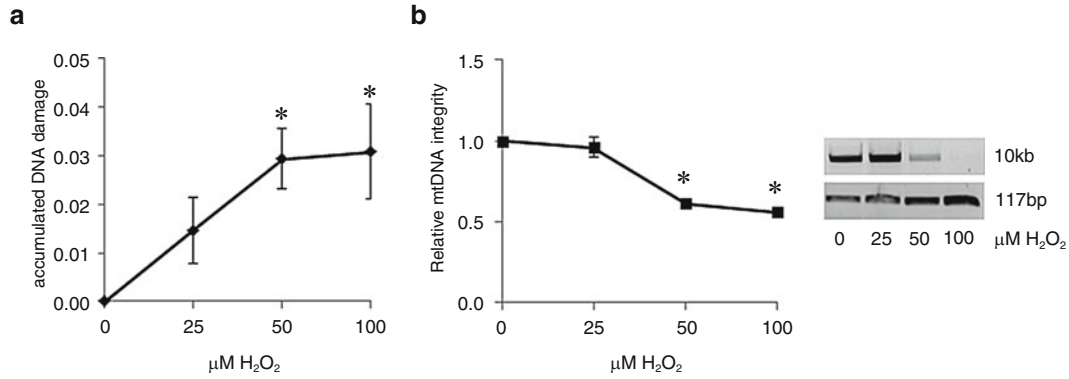

Fig. 1 Validation of the RADF method to detect oxidative DNA damage in mtDNA (*mt-Rnr1*). Mouse embryonic fibroblasts from bl6 mouse grown in DMEM/high glucose were treated with the indicated concentrations of H_2O_2 for 15 min followed by DNA extraction. (**a**) Concentration-dependent damage induction was analyzed by the method described here (Subheading 3.3) and plotted after subtracting background damage. (*b*) Quantitative measurement of mtDNA damage using the alternative damage-mediated inhibition of DNA polymerase method. *Right panel* shows a representative image of amplified short and long fragments. Data are mean ± SEM of three independent experiments.*$p < 0.05$ vs. ctrl

6. The qPCR program is initiated by 94 °C for 10 min followed by 40 repetitions of 94 °C (15 s) and 60 °C (1 min) cycles.

3.3 Damage Frequency Calculation, Verification of Results

1. The resulting ΔCT is calculated (CT-TaqI minus CT-nt), where CT-TaqI and CT-nt represent CT values of TaqI-treated and non-treated genomic DNA, respectively.
2. DNA damage frequency is calculated and presented as $2^{-(ct^{TaqI}-ct^{nt})}$ (*see* **Note 2**).

3.4 Comparison with a Principally Different PCR-Based Method

Cells were exposed to increasing concentrations of H_2O_2 for 15 min in DMEM and the corresponding damage formation was quantified by the RADF method and compared with the damage-mediated inhibition of large fragment amplification method, developed by Ben van Houten and coworkers [8] (Fig. 1).

3.5 Reversible Damage Formation in Nuclear and Mitochondrial DNA, as Detected by the RADF Method

Cells exposed to H_2O_2 for 15 min were grown further in fresh DMEM without oxidant to allow repair. As shown in Fig. 2, nDNA damage was fully repaired within 4 h whereas mtDNA repair was delayed and slower, in line with previous findings [9] (Fig. 2).

3.6 Heterogeneous Types of Damage are Detected by the RADF Method

Cells were exposed to UV light in the CL-100 UV Crosslinker or the methylating agent MMS for 60 min, to induce different types of damage. UV light typically induces bulky lesions that cause DNA helix distortions, while MMS induces base lesions that are less

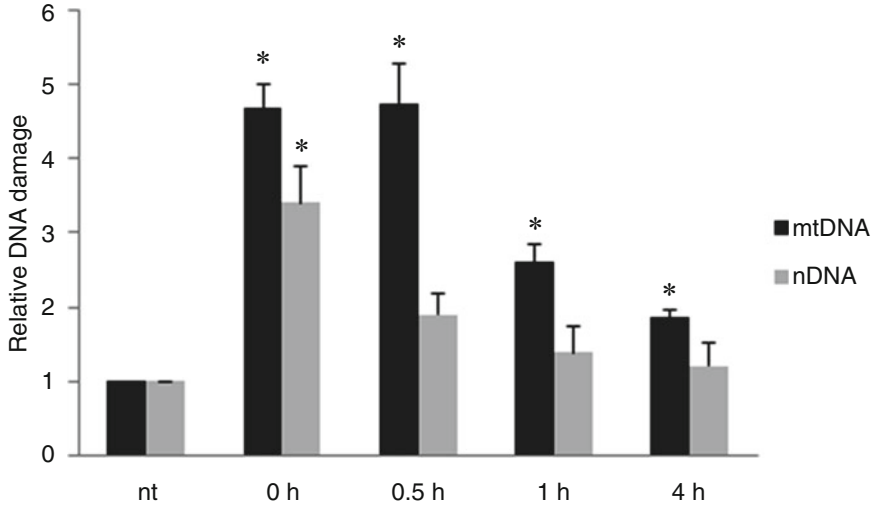

Fig. 2 Induction of damage by peroxide is rapidly repaired in mouse embryonic fibroblasts. Cells were exposed to 50 μM H_2O_2 for 15 min and then allowed to recover in DMEM/high glucose for the indicated times. DNA was extracted, and damage in mtDNA (*mt-Rnr1*) and nDNA (*Ndufa9*) analyzed as described. Data are mean ± SEM of three independent experiments. *$p < 0.05$ vs. nt

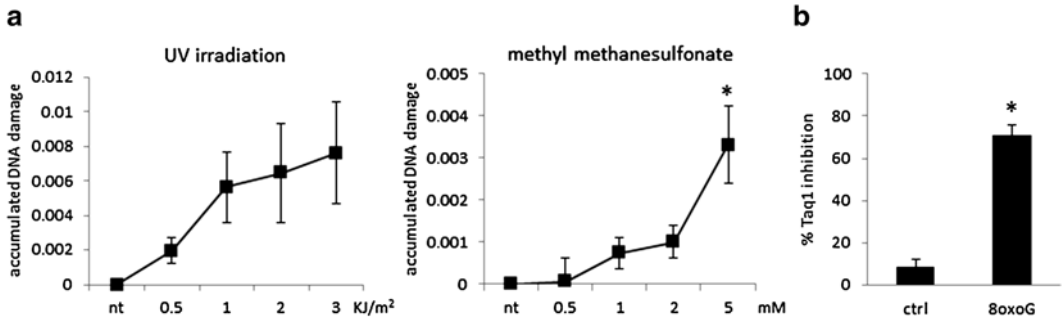

Fig. 3 The RADF method detects damage induced by different genotoxins as well as the abundant 8-oxoguanine. (**a**) Accumulated nDNA damage (*Ndufa9*) in MEF cells exposed to UV irradiation or treated with 0–5 mM MMS for 60 min. (**b**) Evaluation of 8-oxoguanine (8oxoG) to be detected by the RADF method. A synthetic duplex oligonucleotide containing 8oxoG in the recognition sequence was analyzed by the RADF method, and the results presented as relative inhibition of Taq1 cleavage. Ctr: unmodified control duplex. Data are mean ± SE of three independent experiments. *$p < 0.05$ vs control

genotoxic, such as the 7-methylguanine lesion. As shown in Fig. 3a, both UV- and MMS-induced DNA damages are detected by this method. By designing a synthetic oligonucleotide containing the 8-oxoguanine in a defined position, we show that this abundant lesion is also detectable by this method (Fig. 3b) (*see* **Note 3**).

4 Notes

1. Background oxidation of DNA will lead to artificial increased damage frequency and high variations, therefore samples from one experiment to be compared must be isolated simultaneously. Fragmentation of DNA can additionally influence damage analysis. It is therefore recommended to avoid vortexing and harsh pipetting during the DNA isolation procedure.
2. The absolute frequency of lesions calculated is relatively high compared to other methods, and suggest that DNA damage alters DNA topology that secondarily influence restriction digestion efficiency.
3. Since the RADF method requires at least one intact strand to be amplified, it is less suitable for analyzing clustered damage. Thus, we recommend the use of synthetic lesion-containing oligonucleotide (3.6) as positive control rather than in vitro oxidation of DNA by, e.g., H_2O_2/ferrous iron.

Acknowledgements

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Chapter 4

Quantitation of Mitochondrial DNA Deletions Via Restriction Digestion/Long-Range Single-Molecule PCR

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Abstract

Quantification of deletions in mtDNA is a long-standing problem in mutational analysis. We describe here an approach that combines the power of single-molecule PCR of the entire mitochondrial genome with the enrichment of the deletions by restriction digestion. This approach is indispensable if information about wide range of deletion types in a sample is critical, such as in studies concerning distribution of deletion breakpoints (as opposed to approaches where fraction of a single deletion or a limited set of deletions is used as a proxy for total deletion load). Because deletions in a sample are quantified almost exhaustively, the other important application of this approach involves studies where only small amounts of tissue, such as biopsies, are available.

Key words PCR, Mitochondrial DNA, Mutation, Deletions, Restriction digestion, Exercise

1 Introduction

Deletions in mtDNA are detrimental mutations that cause mitochondrial disease and accumulate in various tissues with aging. In addition, deletions are interesting as markers of DNA exposure to various mutagens, such as reactive oxygen species. For example, deletions are increased in some diseases such as Ammon's horn sclerosis, presumably because of increased oxidative stress [1]. Even more subtle interventions, such as prolonged bed rest [2], cause accumulation of mtDNA deletions [3]. Deletions, therefore, are a sensitive indicator of tissue dysfunction, although the precise mechanisms whereby certain interventions or conditions lead to

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the generation (or propagation) of deletions [4] are still debated [5]. On the other hand, in certain cases it is important to be able to demonstrate that levels of deletions are low. This may help to prove that deletions are not involved in certain pathologies [6]. Thus, accurate quantitative analysis of mtDNA deletions is a high priority in research fields ranging from gerontology to environmental health sciences.

In addition to overall frequency of deletions, the distribution of particular types of deletions is also of great interest. Deletions in mtDNA differ in the precise position of the "*breakpoints*"—the positions in mtDNA where DNA had to be cut and rejoined to generate a deletion (Fig. 1). The distributions of the positions of breakpoints differ depending on the source of deletions and may hold clues to the mechanisms responsible for their generation [7–10].

In certain tissues, such as the substantia nigra, deletions accumulate to very high levels, apparently causing cellular dysfunction and possibly functional impairment [11, 12]. In most tissues of normal people, however, deletions are present at low levels, typically below 1 %, though even at low fraction deletions may impact tissue physiology.

There are two main technical challenges in deletion analysis—first, the low percentage of deleted molecules among the wild-type, full-length, molecules and second, the great complexity of deletion mixtures. In most cases a single sample contains dozens of different deletion types, and often, up to thousands or more. Thus it is imperative to separate deletions from the excess wild-type (wt) mtDNA and then isolate individual deletions so they can be sequenced to determine their exact molecular structure, i.e., breakpoints.

Traditionally, mtDNA deletions have been quantified by using long PCR amplification where the primers were set to amplify almost the entire mitochondrial genome (~16.5 kb). Any deleted molecules yield shorter PCR products that can be readily detected by agarose gel electrophoresis (Fig. 1, lower gel). The main problem of this approach is the amplification bias: shorter molecules have a tendency to amplify with much higher efficiency [13]. Thus overall fraction of deletions tends to be vastly overestimated because wild-type PCR products are much longer, typically by a few thousand base pairs, than deleted ones. Moreover, the actual distribution of the different deletions is skewed toward larger deletions/shorter PCR products.

Several approaches address these problems. Some focus on measuring a single type of deletion, such as the most prevalent "common deletion" [14–16], by using deletion- and wt-specific primers in separate PCR reactions. This approach is very precise and sensitive, but does not allow the comparison of the different types of deletions. More importantly, it may be misleading with respect to the total amount of deletions, as the amount of common deletion may not be representative of total mutational load. Indeed,

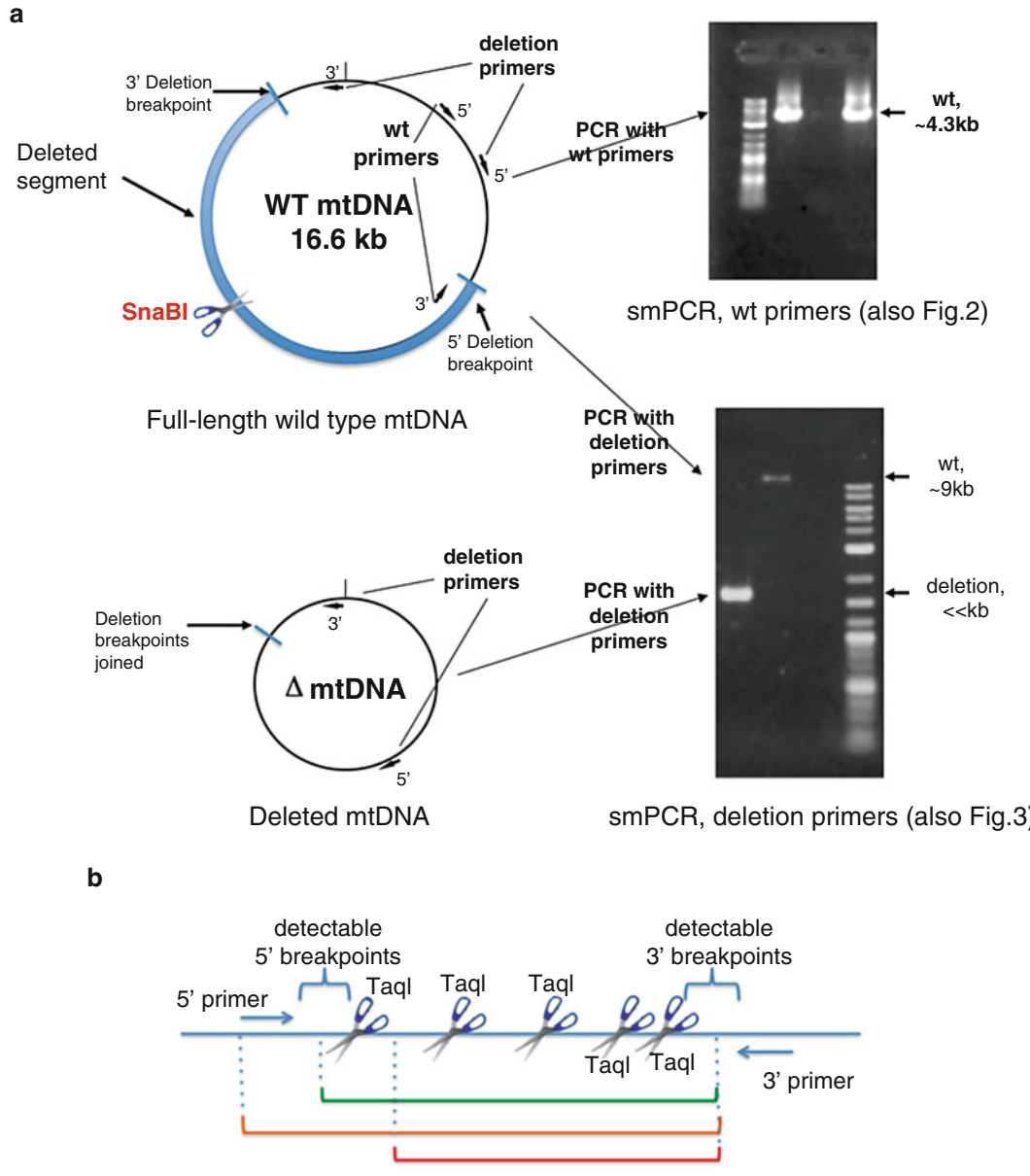

Fig. 1 (**a**) Scheme of long smPCR for quantification of deleted and wild-type (wt) mtDNA. Details are in the text. Note that deletion primers in this example do not cover the entire mtDNA. Using primers with wider coverage is possible, though comes at a cost of more challenging smPCR. See text for further discussion. (**b**) Narrow range of detectable breakpoints in a system with multiple restriction sites. Breakpoints of a detectable deletion must be positioned between a primer and the first restriction site located downstream from that primer. ***Green horizontal bracket***: a detectable deletion. Undetectable deletions: ***red*** (contains one TaqI and will be removed by restriction) and ***orange*** (unamplifiable because removes primer site). Same limitations of detectability of deletions are in effect on the 3′ side (not shown)

the proportion of common deletion among all deletions varies considerably between different samples [9].

To be able to simultaneously quantify essentially any types of deletions we have developed an approach where long PCR of mtDNA is performed in the single-molecule format (smPCR) [17, 18], that is, every PCR reaction contains no more than one deletion molecule, which can be most easily achieved by using diluted samples. Reactions will also contain many wt molecules, which are separated from deletions by gel electrophoresis (*see* next paragraph). By running a large number of independent smPCR reactions, it is possible to count deletions in a sample molecule by molecule. Wild-type mtDNA molecules need to be similarly counted by running PCR at much lower DNA concentrations, where there is about one wild-type mtDNA molecule per PCR reaction. The relative concentration of deleted mtDNA is determined by dividing the number of deletions by the number of wt molecules with appropriate correction for extra dilution used for wt. smPCR reactions can be economically performed in a multiwell PCR plate format (96 wells). This approach has been used, for example, for quantitative analysis of mtDNA deletions in the mtDNA mutator PolG mouse [6].

The smPCR approach solves the problems of deletion detection mentioned earlier: it eliminates competition between different deletions (and the resulting length bias) by separating them into different reactions, and resolves the complexity of deletion mixtures. This approach also solves the problem of the great excess of the wild-type mtDNA (wt). For example, if total deletion fraction is about 1 % (1:100), and smPCR contains about 10 wt molecules per PCR reaction well (this is a realistic example), then each tenth reaction well will contain a deleted molecule. In each of these "positive" reaction wells deletions will be present at about a 1:10 ratio. In our experience, at a 1:10 it is still possible to reliably detect a band corresponding to the deletion by separation on agarose gel. After being detected, deletion needs to be extracted from the gel if it needs to be sequenced for identification of breakpoints. A full 96-well PCR plate will yield about ten deletions.

The problem with this approach is that at lower deletion fractions one needs to increase the number of wells to keep the number of detected deleted molecules at the same level. For example, if the deletion fraction is 0.1 % rather than 1 %, and smPCR is performed at 10 wt molecules per reaction to ensure reliable detection of deleted bands, a whole 96-well PCR plate will yield around one deletion. More plates need to be used to make a reliable measurement. The procedure becomes prohibitively expensive. Increasing the number of wt molecules per well can help, but on the expense of potential loss of some deletions because their bands in the gel become weaker and more difficult to discern from the strong wt bands. Another problem is that identification of deletions involves laborious extraction of deletion bands from gels.

To reduce the number of smPCR wells needed to detect a given number of deleted molecules, one can specifically abolish wt molecules by cutting them with a restriction enzyme at a site located between the primers to prevent PCR amplification (Fig. 1a, SnaBI site) [9]. All molecules with deletions that remove the restriction site will remain amplifiable. In our hands restriction is not complete, with typical efficiency of about 99.9 %, meaning that 1 out of every 1000 wt molecules remains unrestricted. In the above example of a sample with initial concentration of deletions around 0.1 % such a restriction will result in enrichment of deletions to an approximately 1:1 mixture. 1:1 mixture is easier to work with, because wt does not interfere with detection and counting and identification of deletions.

If deletions need to be sequenced, then smPCR should be performed at a concentration of around 1–2 deleted molecule per ten wells. The 96-well plate will then contain 10–20 deletions and 10–20 wt. These numbers are large enough for reliable estimates. Also, most of the deletions will be pure from wild-type and other deletions because it is unlikely that two molecules fall in the same well when there are only 20–40 molecules per 96 wells. Thus deletions will be immediately ready for molecular identification by sequencing (no recovery from gel necessary).

If identification of deletions is not needed and only their frequency is important, then smPCR can be performed at higher molecular concentration, e.g., one or more deletions per well, which will yield about 100 deletions per plate, so that even half or a quarter of a plate will yield a fairly reliable estimate of the number. Deletions in such an experiment are good for counting, but not good for identification, because most will share their well with a wild-type or another deleted molecule.

A downside of the enrichment by restriction digestion is that it narrows the range of deletions that can be detected. The enzyme SnaBI with the site at bp 10,737 (Fig. 1a) was selected because almost all deletions remove this site and are therefore detectable by this approach. However, some deletions do not affect the site and these will be lost for analysis.

Recently, a similar idea (combination of selection of deleted mtDNA by restriction with smPCR) was used to measure deletions with the aid of next-generation sequencing in the Bielas laboratory at Fred Hutchinson Cancer Research Center [19, 20]. In this approach, deletions were enriched by digestion with the TaqI restriction enzyme, which is known for its exceptional efficiency. Unlike most restriction enzymes, TaqI has an efficiency in excess of 99.999 % [21], which essentially allows to completely eradicate wt molecules. The downside of this approach is that the range of detectable deletions with TaqI is much narrower than with SnaBI. TaqI has a large number of sites in mtDNA. Note that

when restriction is used, one can detect only deletions with breakpoints located between each of the two PCR primers and the first restriction site downstream from each of the primers (Fig. 1b). This is also true for SnaBI, but SnaBI has only one site in mtDNA and the two breakpoint "windows" are wide enough to cover almost all possible deletions. Because there are so many TaqI sites, the closest downstream TaqI site is quite close to both of primers, so only sort sequence windows are available for detection of deletion breakpoints. Thus, this method is not right for studying global distribution of deletions across the entire mitochondria genome.

On the positive side, smPCR reactions in this setting can be carried out in emulsion, where every droplet represents a microscopic smPCR reaction. Droplets can be sorted into PCR-positive and PCR-negative groups and then counted. Furthermore, positive droplets can be opened and their content can be sequenced *en masse* by next-generation sequencing, producing detailed distribution of deletion frequencies. This approach allows the sequencing of thousands of individual single molecules. While the above technology is very powerful and elegant, its efficiency is limited by the nature of the typical distribution of deletions in real-life samples. The understanding of this peculiarity of deletion distribution is very important for correct interpretation of the data and for planning of experiments.

An important property of mtDNA deletions is that great majority of deleted molecules in a given piece of tissue originate from a few individual cells with clonally expanded deletions [22], i.e., cells with high percentage of a particular type of deletion. Each of these cells contains only deletions of one type, because they are descendants of a single initial mutant molecule. Different cells contain different types of deletions. As a result, deletions in a tissue mostly come in bunches of deletions of the same type. Thus, if 1000 deleted molecules are sampled, chances are that among those there will be about 100 deletions of one type, about 100 of another and so forth. This means that in the sample there were 10 mutant cells each containing approximately the same number of deletions. This behavior was first observed in skeletal muscle tissue [23] and was subsequently confirmed on the data from Bielas group [24]. This implies that as an increasing number of deleted mtDNA molecules are sampled from a piece of tissue, the types of deletions will soon "saturate," that is, the same types of deletions will be recovered over and over again, making further sampling inefficient as far as the diversity of deletions is concerned. In such case, if more deletion types are needed, it is advisable to use larger sample size. This will hopefully include more cells with different expanded deletions.

Long-range PCR-based detection with restriction is preferred where sample size is limiting, such as in human biopsies. This is

because, in contrast to methods that concentrate on one deletion (such as "common") or a limited set of deletions (such as Bielas approach discussed above), long-range PCR detection of deletions is "exhaustive," that is, it counts essentially all deletions present in a sample. Because deletions usually come in a few bunches of identical deletions originating from single cells with clonal expansions, the chances are high that none of deletions present in a sample will fall within the "limited set," especially if the sample is small. Thus approaches focused on one deletion or a limited set have a high probability of not detecting deletions in a sample even if they are actually there.

In conclusion, restriction/smPCR technique is quite flexible and can be adjusted to a specific task (i.e., mere counting vs. identification of deletions) and to the anticipated fraction of deleted mtDNA in a given tissue sample. In general, molecular identification requires lower smPCR seeding density than mere counting of deleted molecules; lower fractions on deletions can be tackled by using enrichment of deletions by restriction digestion.

2 Materials

1. Fresh frozen or autopsy frozen tissue such as muscle, heart, and brain (*see* **Note 1**).
2. TE buffer: 10 mM Tris–HCl pH 8, 0.5 mM EDTA.
3. Mineral oil. Light mineral oil from a local pharmacy.
4. LongAmp Taq PCR Kit.
5. Ex Taq PCR Kit.
6. SnaBI restriction enzyme; 25,000 μ/mL.
7. NaeI restriction enzyme; 10,000 μ/mL (*see* **Note 2**).
8. 10× restriction enzyme buffer.
9. 96-well PCR fast machine.
10. 12-channel multichannel pipets.
11. Agarose gel electrophoresis equipment compatible with multichannel pipetting.
12. Genomic DNA isolation kit.
13. Pre-chilled razor blades.
14. Primers. Named by numbered position on the 5′-end of reference sequence, F and R for forward and reverse, following by their length.
    (a) "wt primers": for total number of mtDNA molecules.
        - Forward: 1177F34.
        - Reverse: 5463R34.

(b) "deletion primers": for deletions only.

- First stage—Forward: 2999F30.
- First stage—Reverse: 16450R31.
- Second stage—Forward: 3116F39.
- Second stage—Reverse: 16450R31.

3 Methods

3.1 DNA Isolation

1. DNA for analysis is isolated using an appropriate genomic DNA isolation kit.
2. About 10 mg of tissue is shaved from a frozen tissue block with a cold razor blade in a bucket with dry ice.
3. The powder is transferred into a cold, pre-weighed 2 mL tube (a convenient tool for this is a pipet tip) and quickly weighed again to determine the amount of tissue.
4. Lysis buffer is added immediately and DNA isolation is performed according to the kit manufacturer's protocol and final volume adjusted to 100 μL in TE buffer. DNA can be stored at -80 °C for over a year.

3.2 Dilution of DNA Stocks

1. For convenience, the original DNA isolation from Subheading 3.1 is called the "1× stock."
2. Progressively more diluted DNA solutions are prepared using TE buffer. 10× dilutions are typically made by putting 20 μL DNA into 180 μL of TE and gently vortexing in 2 mL centrifuge tubes.
3. The resulting diluted DNA samples are called 10^{-1} for tenfold dilution, 10^{-2} for 100-fold dilution, and so forth (*see* **Note 3**).

3.3 PCR (*See* Notes 4 *and* 5)

1. PCR is typically performed in 15 μL volume reactions overlayered with mineral oil, in 96-well PCR plates. Two different sets of primers are used one for quantifying total number of mtDNA molecules ("wt primers"), and one for deletions only ("deletion primers") (Fig. 1).
2. Thermal profile is biphasic: denaturation at 94 °C, annealing and extension at 68 °C. High annealing temperature ensures specificity but requires using rather stable primers. We recommend ~30 nt primers with about 50 % GC. Extension time depends on the length of the amplicon, and typically is about 1 min per Kb.
3. PCR for total number mtDNA quantification ("wt primers"):

   (a) One stage: 45 cycles, ExTaq polymerase PCR.

   (b) Initial denaturing: 94 °C–1 min.

(c) Cycling: Denaturing—20 s, Annealing and elongation: 68 °C–1 min.

4. PCR for deleted mtDNA molecules ("deletion primers"):
   (a) Two stages (45 cycles and 20 cycles), LongAmp Taq polymerase PCR.
   (b) Initial denaturing: 94 °C–1 min, cycling: Denaturing—20 s, Annealing and elongation: 68 °C–15 min.
5. Because usually one needs to run multiple identical reactions in many wells, PCR master mix is prepared in a larger volume and then aliquoted into individual wells by an automatic pipet, on ice, then over-layered with mineral oil and quickly transferred into pre-heated PCR machine at denaturation temperature to emulate "hot start" PCR conditions.
6. In some cases, two stages of nested or semi-nested PCR are necessary. In such cases, 0.6–1 μL of the First PCR reaction is added to 10–20 μL of a Second PCR master mix kept on ice, then overlaid with mineral oil and transferred into PCR machine in the same way as the First PCR.

3.4 Restriction Digestion

1. Depending on expected deletion load and size of experiment, volume of restriction reaction and concentration of DNA in reaction vary.
2. Example of restriction cocktail for ten reactions: ddH_2O—112 μL, 10× restriction enzyme buffer—24 μL, SnaBI—12 μL, NaeI—12 μL (*see* **Note 2**).
3. Restriction cocktails should be always thoroughly mixed by pipetting. In the example presented, 8 μL of DNA of each sample is pipetted individually in PCR small plates on ice.
4. Restriction cocktail (16 μL) is aliquoted individually followed by pipetting for thorough mixing.
5. Reactions are over-layered with oil and put at 37 °C for 4 h.

3.5 Determining Molecular Concentration

1. To achieve single-molecule concentration of DNA, PCR is first performed in a wide range of sequential dilutions (usually PCR is done in duplicate) of a DNA sample to determine the "critical" concentration at which PCR product "disappears."
2. As a rough guide, a concentration of 1 μL of 10–7 dilution per PCR well will typically yield single molecular pattern of the wild-type molecules.
3. For deletions, 100–1000 higher concentration is usually appropriate, depending on the deletion fraction. Wild-type and deletion PCR are performed using wild-type and deletion primers (approximate positions in Fig. 1, exact sequences above).

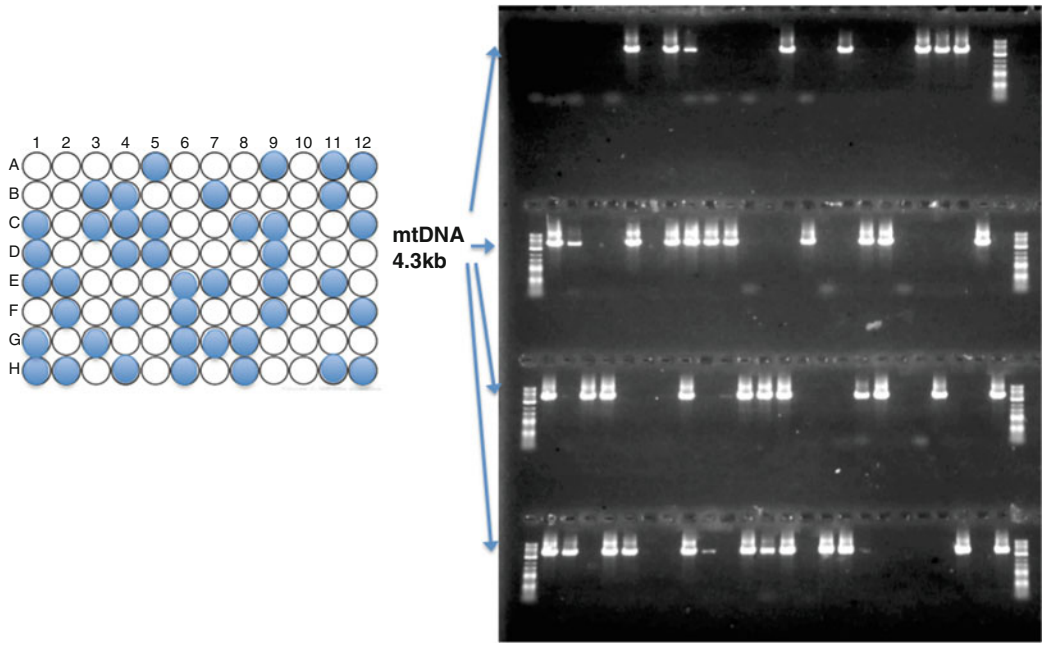

Fig. 2 Determining the molecular concentration of mtDNA. Example of a wild-type 96-well smPCR plate (positive wells marked ***blue***) and the corresponding agarose gel. Note that wells of the 96-well plate were loaded in the order 1A, 21B, 2A, 2B, … There are 42 positive reactions, which implies that there were $-\ln(1-42/96) = 0.58$ molecules per well on average. Assuming that this plate was run at concentration 1 μL of 10–7 dilution per well, we conclude that the concentration in the original 1× sample was $5.8 \times 10 + 6$ molecules per μL

4. Then a pilot PCR is performed at the so determined critical concentration in multiple (e.g., 24) wells, which in most cases results in a single-molecule pattern similar to that shown in Fig. 2.
5. This pattern is used to determine molecular concentration (*see* **Note 6** and Fig. 2).
6. Adjust PCR to an appropriate concentration (about 50 % positive wells for counting or 30 % or less if molecular identification of deletions is needed). Typically three experiments are performed and standard deviations are determined to make sure that error is within desired range.

3.6 Determining Mutant Fractions

1. Mutant fractions are calculated as a ratio of molecular concentration of deletions to that of wild type. To minimize error it is advisable to perform experiments measuring wild-type and deletion concentrations using the same master mix.
2. For example, if anticipated (derived from pilot experiments) fraction of deletions is about 10–3, then after preparing PCR master mix for deletions PCR (omitting primers and polymerase), 1 μL of this master mix can be transferred into 1 mL

of the wild-type master mix, which will set it to a concentration 1000 lower than for deletions, i.e., exactly what is desired.

3. Then appropriate primers and polymerase are added and master mixes are aliquoted into two plates and smPCR run in parallel. With appropriate variations (as needed by the expected ratios and volumes used) this scheme guarantees minimum error in estimates of relative concentrations of wild-type and deleted mtDNA molecules.

4 Notes

1. Tissue/DNA quality is essential. Degraded DNA will show increased bias toward shorter PCR products (larger deleted portions). This is because longer DNA segments have a higher probability to sustain damage (such as nicks and breaks) associated with DNA degradation. How much bias can be tolerated depends on the goals of the particular experiment.
2. NaeI is an enzyme that cuts mtDNA at base pair 933, i.e., outside both wt and deletion PCR fragments. It does not affect the target PCR fragment. We use it to avoid potential intermolecular artificial priming during single-molecule PCR, *see* Ref. [9], supplement, for details. We have not, however, demonstrated that this procedure has any effect on potential artifacts.
3. Diluted DNA samples should be frozen immediately after use at −80 °C. Molecular concentrations in diluted samples are subject to unexplained gradual drift. The impact of this can be minimized by using the same dilution for parallel wt and deletion smPCR as described in step.
4. *smPCR*: *Importance of PCR efficiency*. Any PCR reaction is a complex combination of processes where the desired process (amplification of the target PCR product) competes with various parasitic amplification processes, which include so-called "primer dimers" (visible as low molecular weight "blobs" in some wells in Fig. 2), which are a short amplifiable sequences of unclear origin and structure, sometimes prone to multimerization, which results in unsuccessful PCR and "smears" on the gel. The ability to amplify single molecules depends critically on how well main product competes with parasites. This is particularly important for long PCR fragments, which are naturally more difficult to amplify.

   In general, parasitic processes are slow to form, but parasites are faster to amplify than the main product once they have formed. This implies that contamination of PCR components/samples, plasticware/pipets with even minute amounts of complete PCR reactions may have devastating effect on

smPCR. Critically important is a clean environment, because backward contamination with parasitic PCR products is deadly for smPCR. Therefore the use of a laminar hood, separate room for PCR assembly and tissue/DNA processing, and one-way flow of reagents and consumables is highly recommended. In many cases additional PCR stage with internal primers (nested PCR) may help to increase efficiency, because parasitic fragments usually do not contain internal sites for nested primers. Additionally high PCR efficiency depends on high-quality reaction components including enzymes, "hot start" conditions, fast cycling PCR machine, the use of oil. This is probably because amplification of long-target DNA fragments is more sensitive to all these factors than that of short parasitic species.

5. *Quality control.* Given that there are so many conditions of successful smPCR, one needs a simple test for whether conditions are met. Conveniently, the best test is smPCR itself. Decreasing DNA concentration obviously eventually results in the absence of the target product. In conventional PCR, it is common that with decreasing DNA concentration, the target product becomes gradually fainter and eventually vanishes. In contrast, in smPCR, it is imperative that PCR efficiency and the number of cycles are sufficient to obtain a strong PCR product even when reaction is started from a single molecule. In this case, running many reactions at the "disappearance concentration" yields a digital pattern: some reactions (those where a molecule happened to be) yield strong bands, while others show no product at all. While establishing PCR system it is important to make sure that this "digital" pattern can be reproducibly obtained at the single-molecule concentration.
6. *Calculations of molecular concentration.* When calculating the number molecules in a DNA sample from the results of a single-molecule PCR, it is important to remember that the number of positive reaction wells per plate is not equal to the number of molecules. There are more molecules than positive wells. This is because some of the positive wells contain two or more molecules. These multiple molecule wells are indistinguishable from wells with one molecule. The fraction of wells with two or more molecules is described by the Poisson distribution. Thus Poisson distribution allows to deduce the "real" number of molecules per well from the observed proportion of positive wells. Precisely, the number of molecules per well is equal to $p = -\ln(1-f)$, where f is the fraction of positive wells. For example, if half of the wells are positive, the number of molecules per well is $-\ln 0.5 = 0.69$. Note, however, that when

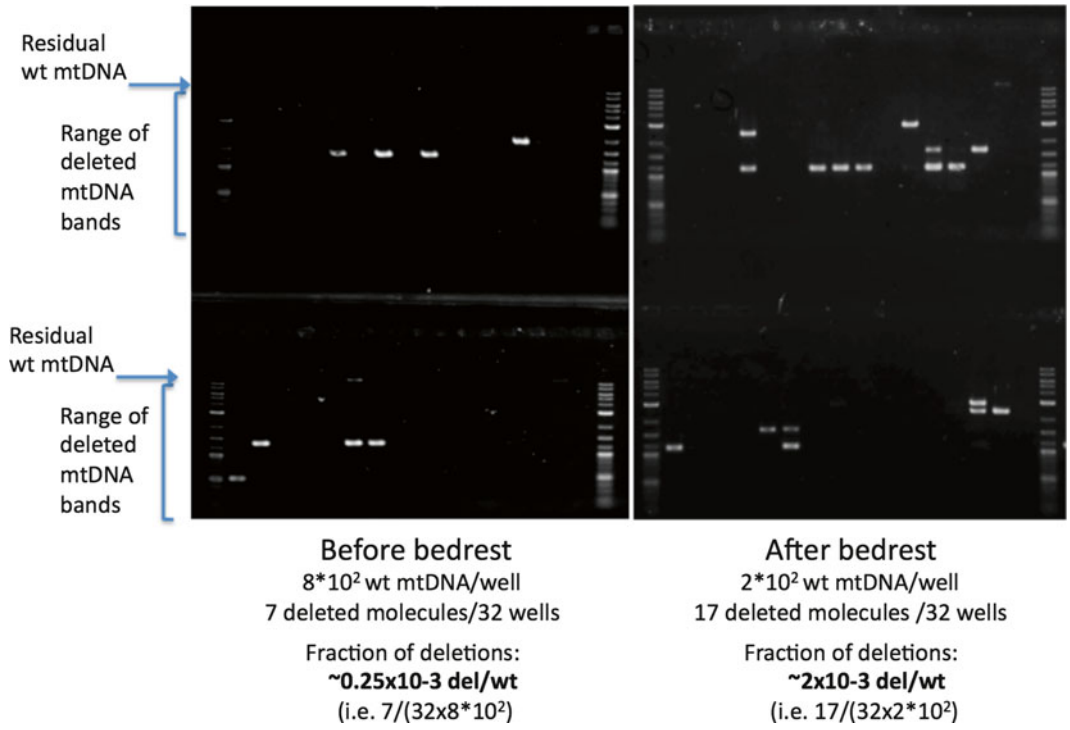

Fig. 3 An example of measuring of deletion fraction in two samples (muscle before and after bed rest) [2]. Only the deletion smPCR gels are shown. Calculations of deletion fractions are explained below the gels; the numbers for the wild type are taken from respective wt gels (not shown). Note that there is no Poisson correction, because different deletions are distinguishable if they appear in the same well

deletions are quantified, Poisson correction is not needed, as long as deletions are different, so that the actual number of different molecules in a PCR well is apparent on the gel (Fig. 3). As a useful rough estimate, a minimum standard deviation of a measurement ("low number error") is equal to the square root of the number of wells (positive or negative, whichever is smaller) counted. A plate with half of the wells positive yields maximum information about molecular concentration.

The actual molecular concentration per μL needs to be calculated back considering the dilution used in PCR (*see* Fig. 2).

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Part II

Visualization of Mitochondrial DNA and Mitochondrial DNA Nucleoids

Chapter 5

A Single-Cell Resolution Imaging Protocol of Mitochondrial DNA Dynamics in Physiopathology, mTRIP, Which Also Evaluates Sublethal Cytotoxicity

Laurent Chatre, Benjamin Montagne, and Miria Ricchetti

Abstract

Mitochondria autonomously replicate and transcribe their own genome, which is present in multiple copies in the organelle. Transcription and replication of the mitochondrial DNA (mtDNA), which are defined here as mtDNA processing, are essential for mitochondrial function. The extent, efficiency, and coordination of mtDNA processing are key parameters of the mitochondrial state in living cells. Recently, single-cell analysis of mtDNA processing revealed a large and dynamic heterogeneity of mitochondrial populations in single cells, which is linked to mitochondrial function and is altered during disease. This was achieved using *m*itochondrial *T*ranscription and *R*eplication *I*maging *P*rotocol (mTRIP), a modified fluorescence in situ hybridization (FISH) approach that simultaneously reveals the mitochondrial RNA content and mtDNA engaged in initiation of replication at the single-cell level. mTRIP can also be coupled to immunofluorescence or MitoTracker, resulting in the additional labeling of proteins or active mitochondria, respectively. Therefore, mTRIP detects quantitative and qualitative alterations of the dynamics of mtDNA processing in human cells that respond to physiological changes or result from diseases. In addition, we show here that mTRIP is a rather sensitive tool for detecting mitochondrial alterations that may lead to loss of cell viability, and is thereby a useful tool for monitoring sublethal cytotoxicity for instance during chronic drug treatment.

Key words Mitochondrial DNA, FISH, Imaging, Metabolism, Transcription, DNA replication, Single-cell, Cytotoxicity, Long-term drug treatment

1 Introduction

Mitochondria play a central role in cellular metabolism and are also involved in cell signaling and cell death. Mitochondria depend on the nuclear genome and on their own genome, thereby requiring continuous and efficient nuclear-mitochondrial communication. This is the case for mitochondrial DNA (mtDNA) replication and transcription, which take place in the organelle, but all mtDNA processing factors are coded in the nucleus [1, 2]. The mitochondrial genome, a double-stranded circular DNA of 16.6 kbp in humans, is present in many copies *per* cell and is organized in

Matthew McKenzie (ed.), *Mitochondrial DNA: Methods and Protocols*, Methods in Molecular Biology, vol. 1351,
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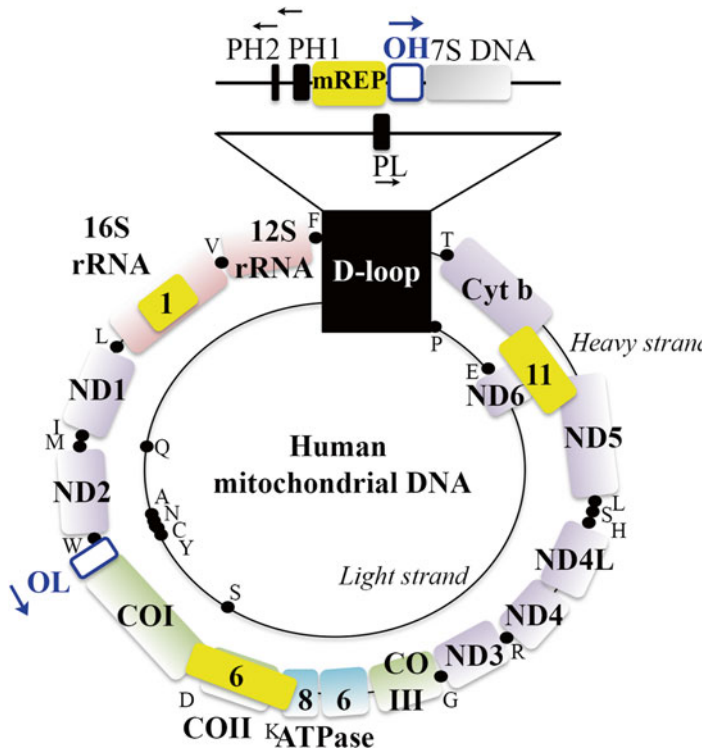

Fig. 1 Human mitochondrial DNA and probes used in mTRIP. Both the H (heavy) and light (L) strands of the mitochondrial genome are indicated (external and inner circle, respectively). The position and name of individual genes are also represented (not at scale), and tRNAs are indicated with a *black dot* and identified with the *corresponding letter*. With the exception of *ND6* and several tRNAs located on the L-strand, all genes are located on the H-strand. The position of probes 1, 6, and 11, which cumulatively constitute the probe mTRANS, is indicated with a *yellow box*. mTRANS targets rRNA as well as several coding genes and tRNA on both strands. Any given mitochondrial transcript or fraction of can be visualized by mTRIP, using the specific probe. The D-loop region, which contains the origin of replication of the H-strand (O_H) and promoters of both H (PH1 and PH2) and L (PL) strands, is zoomed in on top of the scheme; within this region is also shown the position of the mREP probe. The direction of transcription is indicated with a *black arrow* at the level of each promoter; the direction of replication is indicated with a *blue arrow*

multiple protein-containing nucleoids *per* mitochondrion. The human mtDNA codes for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 proteins that function in the respiratory chain (Fig. 1). Mitochondrial RNA is transcribed into polycistronic precursor RNAs and then processed to mature mRNAs, rRNAs, and tRNAs [3]. Therefore, processed and unprocessed RNA molecules may coexist in mitochondria. Distinct models are proposed

for replication of mtDNA [4–7], but it is accepted that replication starts at the O_H origin in the non-coding regulatory region. This generates full-length DNA and also a short molecule, the 7S DNA, that forms a triple-stranded region, the D-loop. The function of 7S has not been elucidated [8].

Mitochondrial replication and transcription have for long time been considered independent from nuclear replication, but recent evidences support a coordination of mtDNA processing with the cell cycle [9–11]. MtDNA replication seems to be heterogeneous among mitochondria and nucleoids. Indeed, super-resolution imaging revealed that mtDNA replication occurs only in a subset of nucleoids [12, 13]. Moreover, we showed a dramatic heterogeneity in mtDNA transcription and initiation of replication in single cells [14], suggesting that the dynamics of mtDNA processing are distinct according to cell type, growth condition, and metabolic state. In this context, the extent and coordination of mtDNA processing may act as markers of changing mitochondrial states and also disease [9, 14].

The dynamics of mtDNA processing can be assessed using mTRIP, an imaging protocol based on modified FISH, that simultaneously detects and quantifies the mitochondrial RNA (mtRNA) content and mtDNA engaged in initiation of replication in single cells [14] (Figs. 2 and 3). Since mTRIP can be coupled to immunofluorescence, proteins are also visualized and their signal quantified. Based on a milder treatment than traditional FISH, mTRIP generally does not damage protein epitopes (or does so mildly), thereby allowing the simultaneous tracking of mitochondrial nucleic acids (DNA and RNA) and proteins.

mTRIP is generally performed using mREP and mTRANS probes. The mREP probe specifically detects mtDNA engaged in initiation of replication through specific binding to a DNA region located upstream of the replication origin O_H in the D-loop (Fig. 1). mREP specifically recognizes target DNA in an open structure conformation, therefore it does not bind to double-stranded mitochondrial genomes that are replication-inactive. The sequence and size of this probe are unique, therefore mREP cannot be substituted with another probe for labeling initiation of mtDNA replication. mREP labeling does not differentiate whether initiation of replication will led to full-length DNA or short 7S DNA.

mTRANS, which detects selected mtRNAs considered to be representative of global mitochondrial transcripts, is a mix of three DNA probes that recognize distinct regions of the mitochondrial genome (Fig. 1). These regions are devoid of major replication origins, include a combination of rRNA, tRNAs, and mRNAs coded on both strands, and are located at regular distances on the mitochondrial genome. Differently from mREP, the mTRANS mix is not unique and any transcript of interest can be specifically

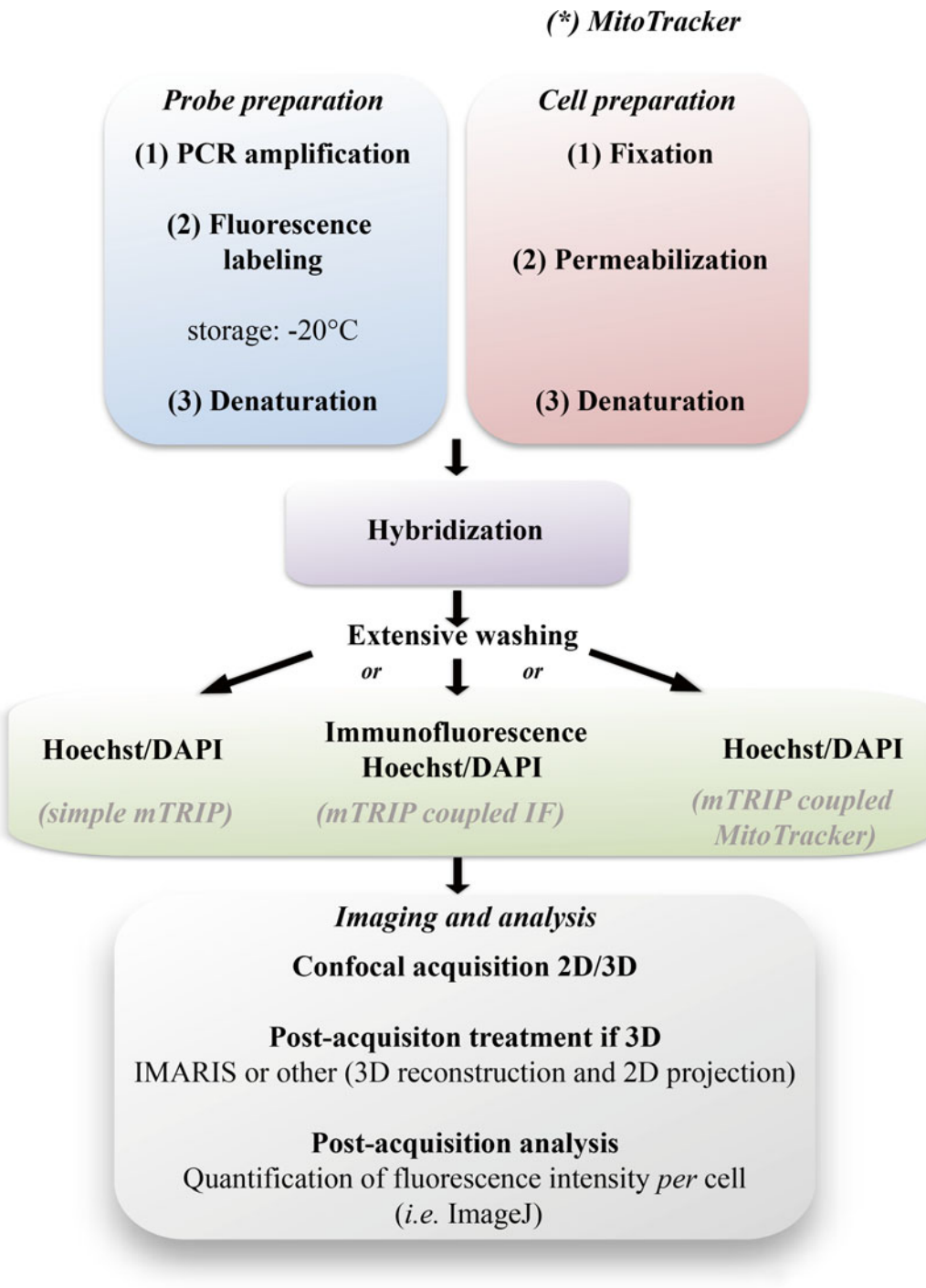

Fig. 2 Scheme of the mTRIP protocol and possible couplings. The key steps of mTRIP labeling are indicated, with preparation of probes and cells shown in parallel. Note that if the MitoTracker labeling is planned (indicated with an *asterisk* in the schema), the compound should be added to the cell culture for the required time, before cell fixation. The following steps until hybridization and washing are common to all procedures. After hybridization and washing, if the mTRIP labeling is done alone (or cells have been previously labeled with MitoTracker), cells must just be stained with Hoechst/DAPI to mark the nucleus. Alternatively, if mTRIP is coupled to immunofluorescence, immunostaining should be performed at this stage, followed by Hoechst/DAPI staining to mark the nucleus. The imaging protocol is common to all procedures; 3D acquisition allows fluorescence quantification of the entire cell volume, but 2D acquisition (and fluorescence quantification) of surfaces on individual sections can also be performed

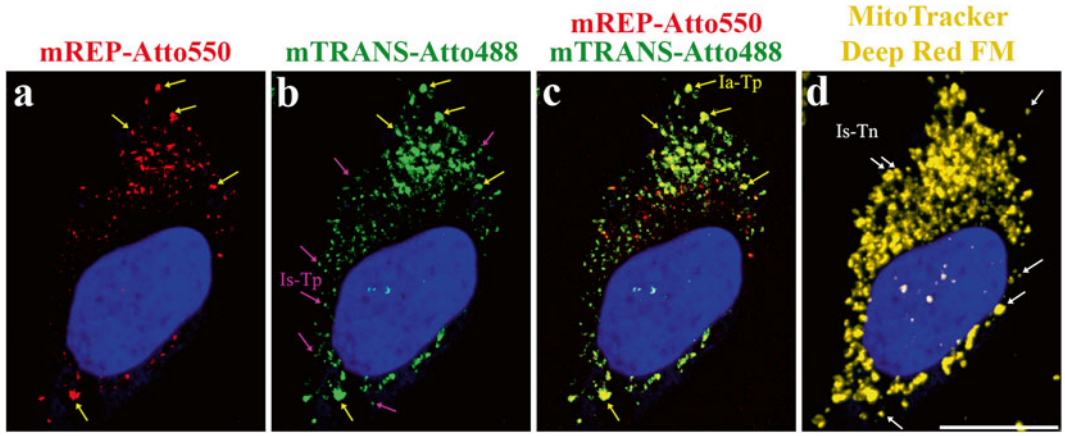

Fig. 3 mTRIP coupled to MitoTracker® Deep Red FM. mTRIP labeling of a human HeLa cell with (**a**) mREP (*red*) and (**b**) mTRANS (*green*) shows mitochondrial DNA initiation of replication and global mitochondrial transcripts, respectively. (**c**) Co-labeling with mREP and mTRANS. (**d**) The mitochondrial network is labeled by MitoTracker Deep® Red FM (*yellow*). While mREP essentially colocalizes with mTRANS (a few $mREP^{+}/mTRANS^{+}$ structures (or Ia-Tp) are indicated with *yellow arrows* in panels **a**, **b**, **c**), independent mTRANS labeling without colocalization with mREP (a few $mREP^{-}/mTRANS^{+}$ structures (or Is-Tp) are indicated with *purple arrows* in panel **b**), as well as mitochondria with undetectable levels of mREP and mTRANS (a few $mREP^{-}/mTRANS^{-}$ structures (or Is-Tn) are indicated with *white arrows* in panels **d**) are also detected. Ia-Tp = Initiation of replication-active and transcription-positive; Is-Tp = Initiation of replication-silent and transcription-positive; Is-Tn = Initiation of replication-silent and transcription-negative. Scale bar = 10 μm

targeted through the design of specific probes (*see* Ref. 14). mTRANS, and any other probe that identifies mtRNAs, will label the target transcripts in the processed as well as unprocessed form.

In a development of this approach, the mitochondrial network can also be tracked. For this, we combined mTRIP with either MitoTracker [15] or immunostaining of a mitochondrial outer membrane protein (TOM22 or TOMM22) [14]. This labeling allows the detection and quantification of at least three classes of mitochondrial subpopulations: (1) replication initiation active and transcript-positive (Ia-Tp); (2) replication initiation silent and transcript-positive (Is-Tp); and (3) replication initiation silent and transcript-negative (Is-Tn) [14]. These mitochondrial subpopulations are highly dynamic and we showed that they vary in number during the cell cycle, under stress, and disease [9, 14]. Thus, isolated or combined mREP and mTRIP labeling (either coupled with labeling of the mitochondrial network or of proteins of interest) act as indicators of the mitochondrial state.

Given the relevance of mtDNA maintenance and expression for the cell function, mTRIP markers may also reveal mitochondrial impairment, eventually associated with cytotoxicity, at an early stage. In Fig. 4 we show that individual mREP and mTRANS labeling detect alteration of mitochondrial parameters at low

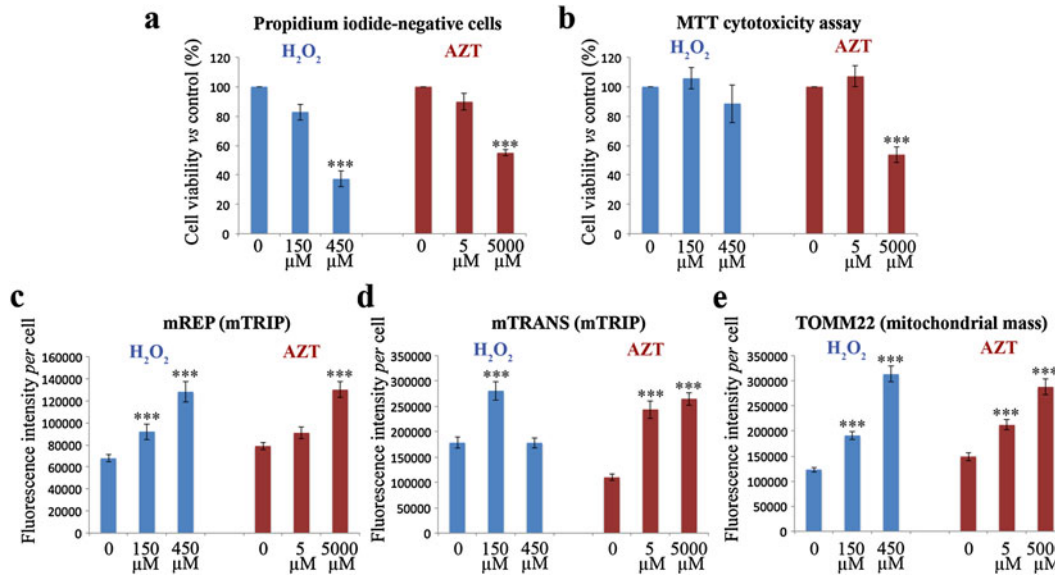

Fig. 4 mTRIP is a sensitive assay for detecting altered mtDNA processing at sublethal doses of H_2O_2 and AZT. As a proof of principle of mitochondrial impairment detected by mTRIP, HeLa cells were treated for 24 h with low and high doses of either the strong oxidizer H_2O_2, which damages cell components and also reduces the mtDNA content [17], or AZT (azidothymidine), an anti-HIV nucleoside analog that causes myopathy after long-term treatment [18]. In parallel, cell viability was assessed, using the propidium iodide (PI) assay and the MTT assay [18]. PI is an intercalating agent that is generally excluded from viable cells, and MTT is a tetrazolium dye that assesses the cellular metabolic activity, which is considered to some extent proportional to cell viability [18], through the levels of NAD(P)H-dependent cellular oxidoreductases. (**a**) Percent of PI-negative (viable) cells in the presence of increasing concentrations of either H_2O_2 or AZT, compared to untreated controls (0). (**b**) Metabolic activity, evaluated with the MTT assay, in cells treated with H_2O_2 or AZT, and expressed as percent of untreated controls (0). (**c**, **d**) Quantification of fluorescence intensity per cell of the mTRIP probe mREP (c) that labels initiation of mtDNA replication, and the mTRANS probe (d) that labels the global mtRNA content. (**e**) Quantification of fluorescence intensity per cell of the immunomarker TOMM22 (or TOM22), a mitochondrial outer membrane protein, used as indicator of the mitochondrial mass [14]. These results show that high doses (450 µM) of H_2O_2 result in a significant loss of cell viability, as expected, with PI assay (panel a) but not with the MTT (panel **b**) assay. Under these conditions, mTRIP displays a large increase of mREP (panel c) but not of mTRANS (panel **d**) signal compared to untreated controls. Similarly, high doses (5 mM) of AZT result in a significant loss of cell viability by PI and MTT assessments, and mTRIP displays a large increase of mREP and mTRANS signal. Taking into account the increase of the mitochondrial mass (immunofluorescence of the outer membrane protein TOM22, panel e), both treatments resulted in globally altered mtDNA processing activity, evaluated by mTRIP. Conversely, low dose (150 µM) of H_2O_2 did not result in loss of viability with either the PI or the MTT assays, whereas resulted in increased mREP and mTRANS signal compared to untreated controls. In a similar situation, low dose (5 µM) of AZT did not affect cell viability, detected by PI and MTT assays, whereas mTRIP resulted in increase of the mTRANS signal. Low doses of AZT did not affect the mREP signal. Taking into account that both treatments also augmented the mitochondrial mass, low doses of either compound resulted in altered mtRNA content and mtDNA initiation of replication. Thus mTRIP reveals alterations of mtDNA processing also at sublethal doses of these cytotoxic compounds. In c–e, quantification was performed on 30 cells per condition, from three independent experiments. Note that mTRIP detects mitochondrial alterations at high and low doses of H_2O_2 and AZT, whereas cytotoxicity, evaluated with standard methods, is detected only at high doses of these compounds. $N = 3$ independent experiments per conditions. $P < 0.001$ (***) *versus* untreated cells (0), according to the *T*-test

doses of the oxidant hydrogen peroxide (H_2O_2) and AZT (azidothymidine, a nucleoside analog used as antiretroviral), below the threshold of cytotoxicity detected with standard tests. As a control, at higher doses of these molecules, standard tests revealed cytotoxicity, and mTRIP detected extensive alterations of mitochondrial parameters. Thus, mTRIP is not conceived to reveal cell death but is highly sensitive to alteration of mitochondrial parameters at sublethal doses of drugs. With these characteristics, mTRIP is of potential interest for evaluating the impact of long-term treatments that affect cell function without immediate risk of cell death.

In conclusion, mTRIP is a powerful tool for fundamental studies of mtDNA processing linked to mitochondrial function, for assessing impaired mitochondrial function in disease, and also evaluating sublethal cytotoxicity.

2 Materials

All solutions must be prepared using ultrapure water. Disposing waste materials must be done according to waste disposal regulations.

2.1 Probe Preparation

1. Lysis buffer to extract total genomic DNA: 0.2 % SDS, 5 mM EDTA, 0.2 mg/mL Proteinase K in 1× phosphate buffered saline (PBS) buffer.
2. *LA Taq* DNA polymerase.
3. Sodium acetate 3 M, pH 5.2, cold ultrapure isopropanol and ultrapure water.
4. Primers for PCR amplification of mTRANS from total genomic DNA:

   mTRANS is a mix of three DNA probes: probe1, probe 6, and probe 11.

   Primers for PCR amplification of DNA probe 1 (961 nt):

   Forward 5′-ACCAGACGAGCTACCTAAGAACAG-3′

   Reverse 5′-CTGGTGAAGTCTTAGCATGT-3′

   Primers for PCR amplification of DNA probe 6 (1,118 nt):

   Forward 5′-CTACCACACATTCGAAGAACC-3′

   Reverse 5′-CGTTCATTTTGGTTCTCAGGG-3′

   Primers for PCR amplification of DNA probe 11 (1,420 nt):

   Forward 5′-CATACCTCTCACTTCAACCTC-3′

   Reverse 5′-TGAGCCGAAGTTTCATCATGC-3′

5. Primers for PCR amplification of mREP from total genomic DNA:

   Coordinates of the primers are according to NC_012920 GenBank.

   mREP (98 nucleotides, nt): coordinates 446–544.

   Forward 5′-ACATTATTTTCCCCTCCC-3′

   Reverse 5′-GGGGTATGGGGTTAGCAG-3′

6. PCR products clean-up system kit.
7. Labeling of purified mTRANS and mREP PCR products. It is strongly recommended to label the DNA probes by Nick-translation using Atto425 or Atto488 or Atto550 or Atto647 NT Labeling Kit (JenaBioscience) (*see* **Note 1**).

Storage of labeled and purified mTRANS and mREP probes: aliquoted at −20 °C in the dark.

2.2 Cell Treatment

1. Tissue culture plates 6-well (12-well or 24-well plates are alternatives for culture of rare primary cells or slow growing cells).
2. Glass cover slips (18 mm diameter or other size, according to diameter of the culture plate).
3. 2 % Paraformaldehyde (PFA) in PBS: mix 5 mL of 16 % PFA with 35 mL of PBS. Store at 4 °C for a maximum of 2 weeks.
4. Permeabilization buffer: 0.5 % Triton X-100 in PBS. Mix 50 μL of 100 % Triton X-100 with 9,550 μL PBS.
5. 20× saline sodium-citrate (SSC) buffer: 3 M NaCl, 300 mM Na_3Citrate.2 H_2O, pH 7.0. For 20× SSC preparation, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of ultrapure water. Adjust the pH to 7.0 with 14 N solution of HCl. Adjust the volume to 1 L with ultrapure water. Sterilize by autoclaving. Store at room temperature for up to 6 months.
6. Pre-treatment buffer for permeabilized cells: 50 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark.
7. Denaturation buffer for permeabilized cells: 70 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark.

2.3 mTRIP Hybridization

1. Parafilm.
2. 100 ng/μL salmon sperm DNA. Dilute stock salmon sperm DNA with PBS. Store at −20 °C.
3. Hybridization buffer: 10 % dextran sulfate/50 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark (*see* **Note 2**).
4. Washing buffer A: 2× SSC in PBS.

5. Washing buffer B: 1× SSC in PBS.
6. Washing buffer C: 0.1× SSC in PBS.

2.4 mTRIP Coupled to Immunofluorescence

1. Parafilm.
2. Blocking buffer: 5 % bovine serum albumin (BSA) in PBS. Store at 4 °C, stable for several weeks (*see* **Note 3**).
3. Primary antibody (*see* **Note 4**). We recommend unconjugated rabbit polyclonal anti-TOMM22 to label mitochondria.
4. Fluorescent conjugated secondary antibody (*see* **Note 5**). We recommend Cy5 conjugated goat secondary anti-rabbit antibody (or another Cy Dye conjugate).
5. Hoechst 33342 or DAPI.

2.5 mTRIP Coupled to MitoTracker®

MitoTracker® Probes (Life technologies). Choose a MitoTracker probe according to spectral characteristics compatible with the successive mTRIP labeling. We recommend MitoTracker® Deep Red (655 nm).

2.6 Imaging Equipment

1. Confocal microscope.
2. Optional for 3D imaging: 3D-reconstruction imaging software.
3. Quantification—imaging software such as Image J, or equivalent.

3 Methods

mTRIP principles are summarized in Fig. 2 and representative mTRIP staining in a human cell is shown in Fig. 3.

3.1 Extraction of Total Genomic DNA

This includes nuclear and mitochondrial DNA. We recommend use of primary cells such as IMR90 at early passages (and not cancer-derived or immortalized cell lines) (*see* **Note 6**).

1. Add 200 µL of fresh lysis buffer to cell pellet.
2. Incubate for 3 h at 50 °C.
3. Add 20 µL of 3 M sodium acetate (pH 5.2).
4. Add 200 µL of cold ultrapure isopropanol.
5. Incubate for 15 min at −20 °C.
6. Centrifuge at 16,100 × *g* at 4 °C for 30 min.
7. Carefully discard the supernatant.
8. Add 500 µL of 70 % ethanol.
9. Centrifuge at 16,100 × *g* at 4 °C for 5 min.
10. Carefully discard the supernatant.
11. The pellet should be transparent if the extraction was correctly done. Dry the DNA pellet for 5 min at room temperature.

12. Resuspend the DNA pellet in at least 100 µL of ultrapure water.
13. Incubate at 4 °C overnight to ensure full resuspension.
14. Quantify the total genomic DNA. Dilute in ultrapure water, if needed.
15. Store at −20 °C.

3.2 Preparation of the Fluorescent DNA Probe

1. PCR amplify the mTRANS probe 1 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
2. PCR amplify the mTRANS probe 6 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
3. PCR amplify the mTRANS probe 11 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
4. PCR amplify the mREP probe using at least 100 ng/µL of total genomic DNA and *LA Taq DNA* polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
5. Purify the PCR products through 2 % agarose gel electrophoresis and then conventional gel and PCR products clean-up kit.
6. Estimate the amount of the purified PCR product. **It is essential to obtain 1 µg of PCR product at a concentration of 75 ng/µL or higher.** If this amount is not reached, the DNA product can be concentrated by sodium acetate and isopropanol precipitation, then resuspended in the appropriate volume of ultrapure water to reach the required concentration. It is nevertheless recommended to repeat the PCR amplification to obtain the appropriate yield rather than concentrate more diluted preparations.

   Be careful, as the following steps are dedicated to DNA probe fluorescent labeling by Nick-translation and are essential for mTRIP. Consider that for mTRIP probes no other technique of DNA labeling than Nick-Translation has been tested to date.
7. Select the fluorescence label for mREP and mTRANS DNA probes (*see* **Note 7**). If mTRIP is coupled to immunofluorescence, or MitoTracker, resulting in four color channels (which include the staining with Hoechst 33342), the recommended choice is: mREP label with Atto550 NT kit, mTRANS label with Atto488 NT kit, immunofluorescence

with a Cy5-conjugated secondary antibody (or, alternatively, mitochondria labeling by MitoTracker® Deep Red FM), and Hoechst.

8. Prepare the mTRANS DNA probe by mixing 333 ng of mTRANS probe 1, 333 ng of mTRANS probe 6, and 333 ng of mTRANS probe 11 (same amount of the three probes) for a final amount of 1 μg DNA, in maximum volume of 14 μL, and in a 0.2 mL thin-wall 8-tube strip with caps for a regular thermal cycler (PCR machine). If the volume is smaller, adjust to 14 μL with PCR-grade water from the kit.
9. Prepare the mREP DNA probe using 1 μg of pure PCR product in a maximum volume of 14 μL, and in a 0.2 mL thin-wall 8-tube strip with caps for a regular thermal cycler (PCR machine). If the volume is smaller, adjust to 14 μL with PCR-grade water from the kit.

The following steps are identical for mREP and mTRANS fluorescence labeling:

10. Gently mix 14 μL of template DNA with 2 μL of 10× NT labeling buffer, on ice.
11. Add 2 μL of Atto488 or Atto550 NT labeling mix.
12. Add 2 μL of 10× Enzyme mix.
13. Gently mix to ensure sample homogeneity.
14. Incubate the mix for 90 min at 15 °C, for example in a regular thermal cycler, in the dark.
15. Add 5 μL of NT Kit Stop buffer to stop the reaction.
16. Transfer the reaction mix to a 1.5 mL tube.
17. Add 2 μL of 3 M sodium acetate at pH 5.2.
18. Add 14 μL of cold ultrapure isopropanol.
19. Gently mix the sample.
20. Incubate at −20 °C for 15 min in the dark.
21. Centrifuge at 16,100 × *g* for 30 min at 4 °C.
22. Discard the supernatant.
23. Add 500 μL of 70 % ethanol.
24. Centrifuge at 16,100 × *g* for 5 min at 4 °C.
25. Discard the supernatant.
26. Add 500 μL of 70 % ethanol.
27. Centrifuge at 16,100 × *g* for 5 min at 4 °C.
28. Discard the supernatant.
29. Resuspend the fluorescence-labeled DNA probe in 50 μL of ultrapure water for a final concentration of 20 ng/μL.
30. Store the labeled DNA probe at −20 °C in the dark.

3.3 Pre-hybridization of the DNA Probe

To reduce bench timing, Subheading 3.3 *(Pre-hybridization of the DNA probe)* can be performed in parallel with Subheading 3.4 *(Preparation of cells on cover slip)*.

1. Selection 1. **ONE probe**, preparation of the reaction mix in a 1.5 mL tube:
   If only one probe (e.g., either mREP or mTRANS) is hybridized, the reaction mix is done in a final volume of 25 μL per cover slip. Gently mix 2 μL of 20 ng/μL fluorescent DNA probe (40 ng DNA probe per cover slip at a final concentration of 1.6 ng/μL) with 4 μL of 100 ng/μL salmon sperm DNA (400 ng of salmon sperm DNA are added for 40 ng of DNA probe, and per cover slip) and 19 μL of hybridization buffer.
2. Selection 2. **TWO probes**, preparation of the reaction mix in a 1.5 mL tube:
   If two probes (mREP and mTRANS) are hybridized, the reaction mix is done in a final volume 25 μL per *c*over slip. Gently mix 2 μL of 20 ng/μL fluorescent mREP probe (40 ng of DNA probe are used per cover slip; final concentration 1.6 ng/μL) and 2 μL of 20 ng/μL fluorescent mTRANS probe (40 ng DNA probe per cover slip; final concentration 1.6 ng/μL) with 8 μL of 100 ng/μL salmon sperm DNA (800 ng salmon sperm DNA are added for 2 × 40 ng of DNA probes and per cove slip) and 13 μL of hybridization buffer.
3. Denaturation: incubate the reaction mixture at 80 °C for 10 min in the dark.
4. Pre-cooling: transfer the tube containing the denatured reaction mix at 37 °C for at least 30 min in the dark. This time can be extended to 1 h maximum (again at 37 °C in the dark). This step helps lowering unspecific binding of salmon sperm DNA *versus* specific binding of DNA probes. Keep the denatured reaction mix under these conditions until the end of Subheading 3.4 (Preparation of cells on cover slip).

3.4 Preparation of Cells on Cover slip

1. Cell culture: culture cells under appropriate conditions on plates that contain a clean slide at the bottom of each well. Ideally, cells should reach no more than 50–60 % confluence to avoid plans with packed cells during microscope analysis.
   If mTRIP is coupled to MitoTracker staining, incubate live cells with MitoTracker® (for instance Mitotracker ® Deep Red) for 1 h under appropriate culture conditions (*see* **Note 8**).
2. Discard the culture medium.
3. Wash cells once with PBS.
4. Add 2 % PFA kept at room temperature.
5. Incubate for 20 min at room temperature (RT).
6. Discard the PFA.

7. Wash two times with PBS. At this step, PFA-fixed cells can be stored in PBS for several weeks at 4 °C.
8. Discard the PBS from (fixed) cells on the cover slip.
9. Permeabilize cells with 0.5 % Triton X-100 in PBS for 5 min at 4 °C.
10. Discard the Triton X-100.
11. Wash four times with PBS.
    OPTIONAL: if required, at this step permeabilized cells can be incubated with specific nucleases for the time recommended by the manufacturer (general recommendation: 1 h at 37 °C). After incubation, wash four times with PBS (*see* Note 9).
12. Discard the PBS.
13. Pre-treat permeabilized cells by adding pre-treatment buffer (50 % formamide/2× SSC in PBS).
14. Incubate for 30 min at room temperature.
15. Discard the pre-treatment buffer.
16. Add 70 % formamide/2× SSC in PBS.
17. Put the culture plate (with, at the bottom of each well, a slide with pre-treated permeabilized cells) on the top of a metal block heater for 5 min at 75 °C, to denature the sample.
18. Immediately transfer the culture plate on ice (0–4 °C) and keep on ice for at least 1 min but not longer than 10 min (*see* **Note 10**).

3.5 mTRIP Hybridization

1. Put a 25 μL drop of denatured and pre-cooled reaction mix which contains DNA probe(s) on an appropriate surface of parafilm (at least 3.6 × 3.6 cm for 18 mm diameter slide). For multiple labeling, use a single surface of parafilm with enough room for multiple and well separated drops.
2. Invert the coverslip upside down, with the side covered by cells (previously denatured sample) directly facing the drop.
3. Incubate at 37 °C for 15 h either on top of a heating metal block in the dark (cover with a non-transparent plastic top to keep dark) or on a humid chamber at 37 °C (always in the dark).

3.6 mTRIP Washing

1. After 15 h of incubation, remove the cover slip from the parafilm surface and put it back into a clean cell culture plate (e.g., a 6-well plate).

   The following steps must be performed in the dark:
2. Wash twice with 2× SSC in PBS for 2 min at RT with gentle shaking.

3. Wash twice with 1× SSC in PBS for 2 min at RT with gentle shaking.
4. Wash twice with 0.1× SSC in PBS for 2 min at RT with gentle shaking.
5. Wash twice with PBS for 2 min at RT with gentle shaking.

At this point, either label the sample by immunofluorescence (step 3.7, mTRIP coupled to immunofluorescence) or do not label the sample by immunofluorescence (this is also the case for MitoTracker labeling) and perform the following:

6. Incubate the cover slip for 1 h in the dark with 10 μg/mL of Hoechst 33342 in PBS.
7. Wash five times with PBS.
8. Mount the cover slip on a clean and dry glass slide using mounting medium or a drop of 50 % glycerol in PBS.
9. Seal the slide with nail polish and keep the mounted slide in a dark and clean box either at RT if confocal analysis is to be done on the same day, or at 4 °C until confocal analysis is done (it is recommended to analyze the slide no later than 2 weeks after labeling).

3.7 Coupled to Immunofluore scence

Follow the immunofluorescence (IF) protocol suggested below (*see* **Note 11**).

1. Incubate the cover slip with 5 % BSA in PBS at RT for 1 h in the dark.
2. Wash twice in PBS for 2 min at RT with gentle shaking.
3. Dilute the primary antibody, anti-TOMM22, 1:200 in 1 % BSA and PBS. We recommend this antibody for assessing the mitochondrial network.
4. Put a 50 μL drop of diluted primary antibody mix on an appropriate surface of parafilm.
5. Put the cover slip upside down, with the side covered by cells directly facing the drop.
6. Incubate at RT for 1 h in the dark. The incubation time depends on the primary antibody used (follow the manufacturer's recommendations).
7. Wash three times in PBS for 2 min at RT with gentle shaking.
8. Dilute secondary antibody (generally 1:1000, follow the manufacturer's recommendations) in 1 % BSA and PBS, and add 10 μg/mL of Hoechst 33342.
9. Put a 50 μL drop of diluted secondary antibody mix on an appropriate surface of parafilm.
10. Put the cover slip upside down, with the side covered by cells directly facing the drop.

11. Incubate at RT for 1 h in the dark.
12. Wash five times in PBS for 2 min at RT with gentle shaking.
13. Mount the cover slip on a clean and dry slide using mounting medium or a drop of 50 % glycerol in PBS.
14. Seal the slide with nail polish and keep the mounted slide in a dark and clean box either at RT if confocal analysis is to be done the same day, or at 4 °C until confocal analysis is done (it is recommended to analyze the slide no later than 2 weeks after labeling) (*see* **Note 12**).

3.8 Imaging Acquisition and Fluorescence Intensity Quantification

1. Acquisition is done with confocal or classical fluorescence microscope (*see* **Note 13**).
2. One can do either 2-dimension (2D) or 3-dimension (3D) acquisition. In the last case, 3D-reconstruction of the image should follow 3D acquisition.
3. If the acquired images are in tiff format (.tiff), the fluorescence intensity per cell can be quantified using Image J software (or equivalent). A 3D-reconstructed human cell stained by mTRIP is shown in Fig. 3 (panels a, b, c).
4. A comparison of mTRIP with propidium iodide (PI) and MTT assays [16] for the detection of sublethal cytotoxicity following H_2O_2 [17] or AZT (azidothymidine) [18] treatment is shown in Fig. 4.

4 Notes

1. Labeling of DNA probes has been tested only with Nick Translation. It is possible that other DNA probes labeling techniques are compatible with mTRIP.
2. The hybridization buffer should be prepared several days (at least 2 days) before the experiment since dextran sulfate takes long time to dissolve.
3. Alternative blocking buffers can also be used.
4. Unconjugated rabbit polyclonal anti-TOMM22 is used to detect the mitochondrial network. However, the mitochondrial network can be detected with other primary antibodies, and immunolabeling can be used to mark other cellular structures or proteins of interest.
5. The choice of the secondary antibody-conjugated fluorescence depends on the available spectra after exclusion of the fluorophore(s) used for DNA probes (mTRIP). All combinations compatible with available fluorophores and the equipment (fluorescence filters) of the microscope can be used.

6. Cancer and immortalized cell lines may have mutated or highly heteroplasmic (coexistence of more than one mitochondrial genome) mitochondrial genomes.
7. The fluorescence wavelengths of the DNA probes can be different from the ones used here. Appropriate fluorophores must be selected *ad hoc*.
8. In this case, use mREP-Atto550 and mTRANS-Atto-488 for mTRIP labeling, and Hoechst.
9. Treatment with nucleases is conceived to identify the DNA or RNA components of the mTRIP signal. DNaseI (recommended 100 U/mL) or RNAseA (recommended 100 μg/mL) treatment identifies the DNA and RNA components of the mTRIP labeling, respectively. Treatment with RNAseH (recommended 100 U/mL) identifies RNA/DNA structures, generally at replication origins and in transcription bubbles. For the combined use of nucleases and the respective readouts on mitochondrial nucleic acids by mTRIP, *see* Ref. 14. Probes mREP and mTRANS have been shown to label only DNA and RNA, respectively [14]. The use of nuclease is recommended for new probes conceived to target a specific region of the mitochondrial genome or a specific transcript.
10. Do not keep denatured samples at RT as this condition will interfere with the hybridization step. Keep the samples always on ice until hybridization.
11. The favorite IF procedure, if different from the one described here, can alternatively be performed.
12. mTRIP can be used with paraformaldehyde-fixed human cells and paraffin-embedded human tissue sections (method not described here).
13. Reliable quantification requires fluorescence measurement within the cell volume. Cell surface (2D-acquisition) can also be acquired instead, but the corresponding quantification will not measure the total cell fluorescence. In case of 2D-acquisitions it is recommended to analyze a high number of individual cells (>50 cells per condition, from three independent experiments), in particular if labeling is not uniform and/or cells have irregular shapes.

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Chapter 6

Scalable Isolation of Mammalian Mitochondria for Nucleic Acid and Nucleoid Analysis

Ken-Wing Lee and Daniel F. Bogenhagen

Abstract

Isolation of mitochondria from cultured cells and animal tissues for analysis of nucleic acids and bona fide mitochondrial nucleic acid binding proteins and enzymes is complicated by contamination with cellular nucleic acids and their adherent proteins. Protocols presented here allow for quick isolation of mitochondria from a small number of cells and for preparation of highly purified mitochondria from a larger number of cells using nuclease treatment and high salt washing of mitochondria to reduce contamination. We further describe a method for the isolation of mitochondrial DNA–protein complexes known as nucleoids from these highly purified mitochondria using a combination of glycerol gradient sedimentation followed by isopycnic centrifugation in a non-ionic iodixanol gradient.

Key words Mitochondria, mtDNA nucleoids, Mitochondrial RNA

1 Introduction

Research on mammalian mitochondrial biogenesis is dependent on the purification of mitochondria in high yield with minimal contamination by non-mitochondrial nucleic acids and proteins. The early efforts to purify mitochondria have been reviewed by Ernster and Schatz [1]. Most modern procedures are based on that of Hogeboom, Schneider, and Palade [2], who used isotonic sucrose solutions to stabilize mitochondria and differential centrifugation to purify them. Mannitol-sucrose-HEPES buffer (MSH) containing EDTA is used in our protocols since mannitol is not metabolized or imported into mitochondria. HEPES is used since it has a better buffering capacity than Tris in the pH 7.5 range and does not interfere with amino group-specific reagents used in some chemical modification procedures. Bovine serum albumin is typically included in isolation buffers to help stabilize mitochondria and to help control proteolysis, but can be omitted when desired for proteomic analyses.

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Investigators have modified these procedures to selectively remove the contaminants of greatest concern to their research. For example, in membrane research it is most important to remove microsomal contaminants derived from the plasma membrane or endoplasmic reticulum, capitalizing on the greater density and faster sedimentation of mitochondria. Preparation of mitochondrial ribosomes required multiple wash steps to remove cytosolic ribosomes [2, 3]. In nucleic acid research, the greatest concern is contamination with non-mitochondrial nucleic acids. Removal of these contaminants by treatment of mitochondria with nucleases has been used routinely [4].

Numerous publications on mitochondrial DNA (mtDNA) have used hypotonic cell disruption and differential sedimentation, often with sucrose density gradients, to purify mitochondria. Based on extensive experience with such procedures [5], we have identified a number of problems requiring adjustments, as follows: (1) Hypotonic lysis can result in mitochondrial swelling, often with membrane damage or rupture; (2) Mechanical disruption of small quantities of cells and/or of cells with sparse cytoplasm may be inefficient; (3) Nuclear disruption and the release of chromosomes from mitotic cells leads to artifactual adherence of nuclear DNA on the surface of mitochondria; (4) This procedure does not remove cytoplasmic RNA and ribosomes that bind to the mitochondrial outer membrane, due to either physiological association of ribosomes or to artifactual causes [6].

Here, we provide protocols that we have found to be effective for rapid preparation of mitochondria for nucleic acid analysis in a wide variety of settings. The basic approach employs treatment of cells with a low concentration of digitonin to permit facile cell lysis under isotonic conditions, basically as described [7]. Following removal of nuclei by two or more successive sedimentation steps, mitochondria are pelleted and washed with buffer containing 1 M KCl. Figure 1 shows that mitochondria prepared **without** a high salt wash contain abundant nuclear DNA (nuDNA) contaminants. Treatment of mitochondria with DNase I degrades this contaminating nuDNA, as noted by Higuchi and Linn [8] but leaves oligonucleosome-size fragments associated with mitochondria. Importantly, these contaminating fragments may not be retained on agarose gels, giving a false impression that the nuDNA has been removed. In our view, the failure to appreciate this has led to great confusion over the incidence of damaged residues in mtDNA, since many publications have failed to remove nuDNA effectively. In the larger scale Method 2 we suggest incubating mitochondria during nuclease treatment in a buffer that supports respiration and enzyme activity, adapted from [9]. Our proteomic analyses have shown such nuclease-treated mitochondria still contain considerable amounts of nuclear histones and cytoplasmic ribosomal proteins (data not shown). Washing mitochondria with mannitol-sucrose buffer

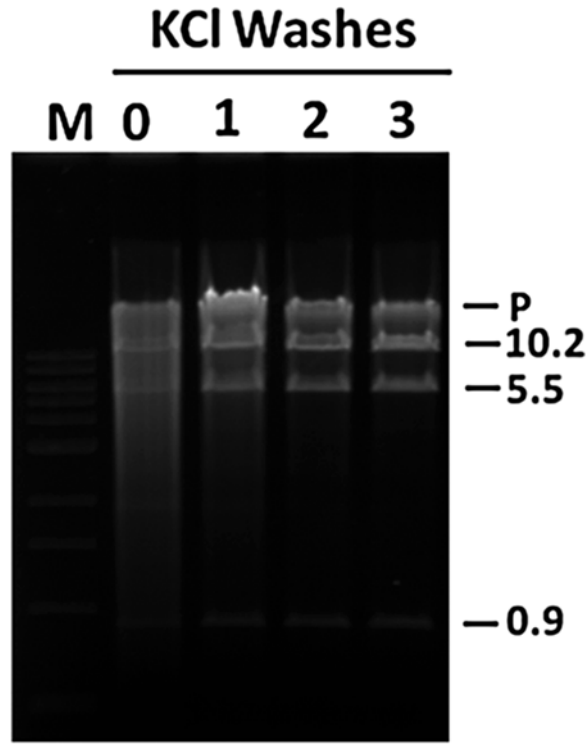

Fig. 1 HindIII digest of purified HeLa mitochondrial DNA shows the removal of nuclear DNA contaminants with successive 1 M KCl washes, without nuclease treatment. ***Lane M*** contains a 1 kB ladder of DNA mobility markers and other lanes are designated by the number of 1 M KCl washes employed. The sizes of mtDNA fragments are shown in kB, along with a partial digest product (***P***)

containing 1 M KCl removes a substantial fraction of nuDNA and cytoplasmic rRNA. Figure 2 shows that this treatment also minimizes contamination by a cytoplasmic ribosomal protein. Several important caveats apply here. First, it is generally easier to prepare highly purified mitochondria from some tissue sources than from cultured cells. Liver mitochondria, for example, are easy to prepare since they occupy 16 % of the volume of hepatocytes and nuDNA contamination is reduced since the mitotic index is very low. Second, mitochondria are highly dynamic organelles that engage with other organelles, such as endoplasmic reticulum, interact with cytoplasmic enzymes under normal physiological conditions, and host cytoplasmic ribosomes participating in co-translational import of certain mitochondrial proteins. Therefore, it is impossible to prepare "pure" mitochondria. In cases where cell fractionation is used as a criterion to show that a particular protein is an authentic component of mitochondria, it is necessary to show that it is increasingly enriched as mitochondria are more highly purified. This concept has been adapted in high-throughput proteomic studies as protein correlation profiling [10] or subtractive proteomics [11].

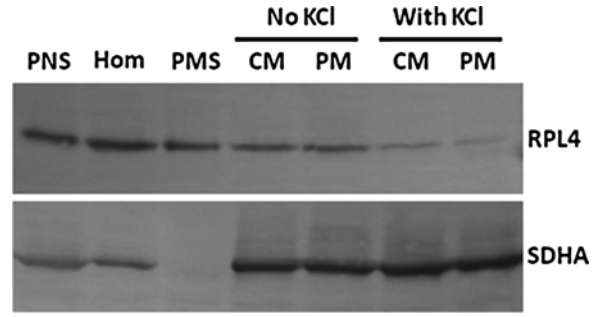

Fig. 2 HeLa cells fractionated either without or with one wash using buffer containing 1 M KCl, producing crude mitochondria (***CM***) which were then further purified on a 1.7 M/1 M sucrose step gradient (PM). 10 μg of protein were loaded in each lane of a 12 % SDS polyacrylamide gel. Proteins were immunoblotted for RPL4, a cytosolic ribosomal protein, or SDHA, a mitochondrial protein subunit of Complex II. ***PNS*** post-nuclear supernatant, ***Hom*** whole-cell homogenate, ***PMS*** post-mitochondrial supernatant

In this article, we present three protocols to prepare mitochondria and mtDNA nucleoids. Method 1 permits rapid preparation of mitochondria from small quantities (e.g., one to three 10 cm tissue culture dishes) from several samples handled in parallel. Method 2 is a scaled-up version that employs nuclease treatment and additional sucrose gradient sedimentation to obtain more highly purified mitochondria. Examples of the application of these methods are found in [12–14]. We have used these methods for a variety of cultured cells (HeLa, A549, H358, normal human fibroblasts, mouse 3T3) as well as mouse liver and Xenopus oocytes.

Method 3 uses mitochondria prepared with Method 2 for preparation of mtDNA nucleoids. This consists of a procedure for lysis of mitochondria with the non-ionic detergent Triton X-100 and a two-dimensional centrifugation protocol to prepare nucleoids based on their large size and buoyant density. In the first dimension, a 15–40 % glycerol gradient is used to separate large nucleoids from smaller complexes, including mitoribosomes. We had previously used sedimentation for 1.5 h to separate slowly and rapidly sedimenting nucleoids [15]. In that study, we reported that the rapidly sedimenting nucleoids are more closely associated with cytoskeletal proteins. In Method 2, we employ a more lengthy 4 h centrifugation step to permit both forms of nucleoids to sediment to a pad containing 20 % glycerol/30 % iodixanol in order to facilitate their rapid collection and further analysis using isopycnic centrifugation in a non-ionic iodixanol gradient. This second separation takes advantage of the density of nucleoids with their associated proteins and, as an equilibrium method, is not strictly time-sensitive. We routinely prepare and lyse mitochondria and complete the glycerol gradient in a single day without exposing material

to freezing and thawing. The iodixanol gradient is then run overnight for 14–16 h. We reported that most proteins shown to function in mtDNA replication and transcription remain associated with the mtDNA through both centrifugation steps, including TFAM, mtSSB, mtDNA polymerase γ, mtRNA polymerase, and proteins involved in mtRNA processing and early stages in ribosome assembly [14]. This final fraction also contains mitofilin and other components of the MICOS complex [16] as well as cytoskeletal proteins. MICOS complexes and cytoskeletal structures are much more abundant than nucleoids, so that it is difficult to determine if the apparent association of these proteins with nucleoids is biologically significant. Others have reported a role for actin and myosin in mtDNA maintenance [17]. Hopefully, the methods presented here will help to advance understanding of mtDNA nucleoid dynamics.

2 Materials

1. Trypsin–EDTA and Trizol.
2. 10 % (wt/vol) digitonin in DMSO (20 % inert); store 200 μL aliquots at 4 °C.
3. Mannitol.
4. 1 M 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0, dissolved in water, autoclaved; store at room temperature.
5. 0.25 M ethylenediaminetetraacetic acid (EDTA)–NaOH pH 8.0, dissolved in water, autoclaved; store at room temperature.
6. 0.5 M (wt/vol) dithiothreithol (DTT), dissolved in water, filter sterilized; store 1 mL aliquots at −20 °C to avoid oxidation.
7. 4 mg/mL radioimmunoassay grade bovine serum albumin (BSA) (US Biochemical) in H_2O; sterilized by filtration through a 0.22 μm membrane; store at 4 °C.
8. 3 M KCl in H_2O; autoclaved; store at room temperature.
9. 200 mM phenylmethanesulfonylfluoride (PMSF), dissolved in isopropanol; store at room temperature.
10. 5 mg/mL leupeptin in H_2O; store 500 μL aliquots at −20 °C.
11. 1 mM pepstatin A dissolved in methanol; store at −20 °C.
12. 0.4 % trypan blue in PBS.
13. Glutamic acid, sodium malate, adenosine diphosphate, sucrose, and ultrapure glycerol.

14. 2 mg/mL DNase I Type II; dissolved in 10 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 40 % glycerol; store at –20 °C.
15. 1 mg/mL RNase A (Type IA) in 10 mM NaAcetate, 50 mM HEPES, pH 8.0, 40 % glycerol; store at –20 °C.
16. 10 % Triton X-100.
17. Turbo DNase (*Serratia marcesens* nuclease; either Benzonase (EMD) or Turbo DNAse (Accelagen)); store at –20 °C.
18. Iodixanol 60 % solution.
19. SYBR Green I and II dyes.

2.1 Equipment

1. Inverted light microscope (10× and 40× objectives) with microscope slides and cover glass.
2. Hemocytometer or alternate cell counting device.
3. Refrigerated swinging bucket centrifuge.
4. Super-speed refrigerated centrifuge with swinging bucket rotor and polycarbonate tubes.
5. Ultracentrifuge and polyallomer tubes.
6. 1 and 3 mL syringes with 25-G needle.
7. Gradient forming device.

2.2 Reagent Stock Preparation

NOTE Prepare all reagents prior to starting the procedure to avoid delays.

For all buffer solutions, labile ingredients such as 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin and 1 μM pepstatin A are added from stock solutions shortly before use.

1. 2.5× MSH Buffer (2.5× Mannitol-Sucrose-Hepes, pH 8.0-EDTA): 525 mM mannitol, 175 mM sucrose, 50 mM Hepes, pH 8.0, 5 mM EDTA. Stock solution filter sterilized and stored at 4 °C.
2. 2.5× MSH/$MgCl_2$: 2.5× MSH Buffer with 5 mM $MgCl_2$ substituted for EDTA.
3. Mitochondrial Isolation Buffer (MIB): 1× MSH, 0.2 mg/mL BSA, 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin, 1 μM pepstatin A.
4. Potassium-containing MIB (KIB): MIB supplemented with 1 M KCl.
5. MIB/Mg: MIB supplemented with 10 mM $MgCl_2$.
6. Sucrose Gradient Buffers: 1.7 M or 1 M sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA. Stock solutions filter sterilized and stored at 4 °C. Supplement with 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin, 1 μM pepstatin A just before use.

7. 1× Nuclease Buffer (5 mL): 2 mL 2.5× MSH/Mg, 1.25 mL 4× Substrate mix (see below), 0.1 mL 3 M KCl (60 mM final), 50 µL 1 M $MgCl_2$ (10 mM final), 10 µL 500 mM K_2HPO_4 (1 mM final) and 1.59 mL water. Supplement with 2 mM DTT, 0.2 mM PMSF, 5 µg/mL Leupeptin, 1 µM Pepstatin A just before use.
8. 4× Substrate mix (25 mL): 4 mM ADP, 20 mM glutamic acid, 20 mM Na_2-malate, 1 mg/mL BSA. Dissolve powders in about 20 mL of water, neutralize the organic acids with 200 µL 5 M NaOH, add sterile-filtered BSA in solution and adjust to 25 mL. If BSA is exposed to the acids without neutralization, a precipitate develops. Filter sterilize and store frozen aliquots of 1.25 mL.
9. 5× Mitochondrial Lysis buffer: 100 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.2 mM PMSF, 5 µg/mL Leupeptin, 1 µM Pepstatin A.
10. Glycerol Gradient Buffers (12 ml each).

15 %	40 %
2.25 mL 80 % glycerol	6 mL 80 % glycerol
2.4 mL 5× lysis buffer	2.4 mL 5× lysis buffer
0.24 mL 10 % TX-100	0.24 mL 10 % TX-100
7.11 mL H_2O	3.36 mL H_2O

11. 20 % Glycerol/30 % iodixanol Pad: 500 µL of 1:1 mix of 40 % glycerol gradient buffer and 60 % Iodixanol.
12. Iodixanol Gradient Buffers (12 mL each).

20 %	40 %
4 mL 60 % iodixanol	8 mL 60 % iodixanol
2.4 mL 5× lysis buffer	2.4 mL 5× lysis buffer
0.24 mL 10 % TX-100	0.24 mL 10 % TX-100
5.36 mL H_2O	1.36 mL H_2O

13. TE buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA.

3 Methods

3.1 Method 1. Small-Scale Mitochondrial Preparation with KCl Wash (See Note 1*)*

Note on scale: This protocol is based on packed cell volume (PCV) under 200 µL from 1 to 3 10-cm plates. The emphasis on rapid purification and the ability to handle multiple samples in parallel with no sucrose gradient step provides slightly lower purity than Method 2. Brief RNase treatment replaces DNase treatment as in Method 2. With KCl wash, cytoplasmic RNA contamination is minimal.

1. Trypsinize cells and stop trypsin with complete medium as usually performed in tissue culture. Centrifuge the cell suspension at 600×*g*, 5 min, rinse with PBS and repellet cells. All steps should be on ice or 4 °C unless otherwise indicated.
2. Resuspend cells in 1 mL MIB. Transfer to a microfuge tube.

   Add 3 μL 10 % digitonin in DMSO. Gently invert the suspension several times, then check cell permeability with trypan blue by mixing 3 μL of cells with 3 μL Trypan blue stock. >90 % of cells should be stained with trypan blue (*see* **Note 2**).
3. Add MIB to 1.5 mL to decrease the digitonin concentration; repellet cells this time at 900×*g*, 5 min in adapters in a swinging bucket rotor.
4. Remove the supernatant, resuspend the pellet in 0.8 mL MIB. Homogenize by passage through a 25 G needle fitted to a 1 mL syringe. About ten "firm" passages with minimal frothing should suffice. Confirm cell breakage under the microscope and add more passages as needed (*see* **Note 5**).
5. Add 0.8 mL MIB and centrifuge at 1,200×*g*, 5 min to pellet nuclei and unbroken cells.
6. Withdraw the supernatant to a new tube, avoiding the pellet, and repeat the nuclear sedimentation.

   The final supernatant is referred to as the "post-nuclear supernatant," PNS. Microscopic examination should reveal no nuclei. If nuclei are present, repeat the centrifugation.
7. Pellet mitochondria by centrifuging in a refrigerated microfuge at 15,000×*g*, 10 min.
8. (a) *For protein only; if cytoplasmic RNA is not an issue:*
   Resuspend the mitochondrial pellet in 1 mL KIB; repellet mitochondria as above.
   (b) *If RNAse treatment is desired:*
   Resuspend the mitochondrial pellet with 400 μL MIB/Mg supplemented with 4 μL 1 mg/mL RNase A and 0.5 μL Turbonuclease. Incubate at room temperature for 15 min. Add 1 mL KIB, mix and repellet mitochondria as above.
9. Resuspend mitochondrial pellet with 500 μL MIB and repellet. If samples are for analysis of mitochondrial proteins, use MIB lacking BSA.
10. Process the mitochondrial pellet for RNA using Trizol if desired; for protein analysis, either store the pellet frozen or resuspend in sample loading buffer.

3.2 Method 2. Medium/Large-Scale Mitochondrial Preparation with Nuclease Treatment and Sucrose Gradient

1. *Preparation of buffers.* The reagents in this protocol are scaled for 2×10^8 cells, which can be cultured from twelve 150 mm × 25 mm tissue-culture plates.

   Prepare 30 mL MIB, 5 mL KIB, 1 mL Nuclease Buffer, 5 mL 1× MSH Buffer and 5 mL 0.5× MSH Buffer. All buffers should be cooled on ice and all steps performed at 4 °C unless otherwise indicated.

2. *Collect cells.* If working with adherent cells, harvest cells by scraping or with trypsin–EDTA, then neutralize the trypsin with media containing serum. If working with suspension cells, omit the trypsin step. Count the cells with a hemocytometer. Collect the mammalian culture cells into a suitable centrifuge tube and pellet the cells by centrifugation at 500 × *g* for 5 min.

3. Remove the media and resuspend the cells in 5 mL PBS. Pellet the cells again, then resuspend the cells in 3 mL MIB and transfer to a 17 × 100 mm tube (*see* **Note 3**).

4. *Digitonin treatment.* Add 10 % digitonin until all of the cells are permeable to trypan blue staining. Initially add 12 μL digitonin to the cells. Mix 3 μL cell suspension with 3 μL trypan blue, and check for complete permeabilization by microscopy. If necessary, add additional small increments of 2–4 μL 10 % digitonin until >90 % of cells are stained (*see* **Note 2**).

5. Add 7 mL of MIB to dilute the concentration of digitonin in the cell suspension. Centrifuge the cells at 1,200 × *g* for 5 min (*see* **Note 4**).

6. *Cell disruption.* Resuspend the cells in 3 mL MIB and pass through a 25-G needle 8–10 times. Inspect a sample volume for lysis using a microscope (*see* **Note 5**).

7. *Pellet nuclei.* Add 5 mL of MIB to the lysate to increase the volume of lysate and to reduce the amount of mitochondria trapped in the nuclear pellet. Centrifuge the lysate at 900 × *g* for 5 min. Collect the supernatant (post-nuclear supernatant, PNS) into a new 15 mL conical tube by pipetting, not by decanting, to avoid nuclear contamination. Repeat the centrifugation, withdraw the supernatant to a fresh tube and check a small sample under the microscope (*see* **Note 6**).

8. *Pellet mitochondria.* Transfer the post-nuclear supernatant to a tube compatible with an HB-6 rotor or equivalent. To obtain crude mitochondria, centrifuge at 16,000 × *g* for 15 min. Remove the supernatant (post-mitochondrial supernatant).

9. *KCl wash.* Resuspend the crude mitochondrial pellet in 3 mL KIB and centrifuge in the HB-6 rotor as before (*see* **Note 7**).

10. *Nuclease treatment.* Discard the supernatant. Resuspend the high-salt-washed mitochondria in 1 mL Nuclease Buffer, transfer to a microfuge tube and add 20 μL of 2 mg/mL

DNaseI and 1 μL Turbonuclease. Incubate in a 37 °C water bath for 20 min. RNase may be included if removal of cytoplasmic RNA is desired. Add 100 μL 0.25 M EDTA to stop DNase activity.

11. *Sucrose gradient centrifugation*. While nuclease treating the mitochondria, prepare a sucrose step gradient. Add 3 mL of 1.7 M sucrose buffer to the bottom of an SW-41 tube (14×89 mm) and carefully layer 6 mL of 1 M sucrose buffer on top. Formation of this sucrose step gradient should be performed by using 5 mL serological pipettes and an electronic pipetter on the slowest speed. Carefully layer the mitochondria on top of the sucrose step gradient. Centrifuge in a Beckman ultracentrifuge with an SW-41 swinging bucket rotor at 77,000×*g* (25,000 rpm) for 25 min (*see* **Note 8**).
12. *Collect mitochondria*. Mitochondria will form a light brown layer at the 1.7/1 M sucrose interface. Carefully aspirate the upper solution which contains some residual nuclease. Collect the purified mitochondria from the sucrose interface and dilute it with 2–3 volumes of 0.5× MSH to reduce the sucrose concentration sufficiently (*see* **Note 9**). This suspension can be distributed into microfuge tubes. Pellet the mitochondria at 10,000×*g* for 10 min and then remove the supernatant. Mitochondria may be frozen at –80 °C at this point or directly taken into further steps for analysis (*see* **Note 10**).

3.3 Method 3. Two-Dimensional mtDNA Nucleoid Preparation

1. *Mitochondrial lysis*. Remove the supernatant from the final mitochondrial pellet and add 200 μL 5× lysis buffer and an appropriate volume of H_2O to adjust the sample to a volume of 800 μL. Add 200 μL 10 % Triton X-100 to final 2 % concentration. Incubate on ice with gentle mixing for 15 min. Pellet insoluble debris in a microfuge tube at 5,000×*g* for 5 min.
2. *First dimension separation on a glycerol gradient*. Carefully layer the cleared lysate on a pre-formed 10 mL 15–40 % glycerol gradient in lysis buffer over a 500 μL pad of 20 % glycerol/30 % iodixanol in an SW41 centrifuge tube (*see* **Note 11**). The gradient can be generated using a gradient-forming device or by layering several zones of solutions containing progressively lower glycerol concentrations. Centrifuge for 4 h at 151,000×*g* (35,000 rpm in an SW41 rotor).
3. *Collect fractions*. The 4 h glycerol gradient is designed to permit nucleoids to sediment to the pad. The glycerol gradient is fractionated by withdrawing 14×750 μL samples successively from the top of the gradient (*see* **Note 12**).
4. *Detect mtDNA*. Nucleic acid is detected in gradient fractions using SYBR Green II staining in microtiter dishes (*see* **Note 13**). Mix 3 μL of fraction with 100 μL of 1:10,000 SYBR Green II diluted in TE buffer. Fluorescence is detected using a

Typhoon scanner (GE Healthcare) or a fluorescent plate reader. Most free proteins and tRNA are found in the top 3–4 fractions. Ribosomal subunits and many mRNAs are in the center of the gradient and can be recovered for analysis as desired.

5. *Second dimension purification of nucleoids on an iodixanol gradient.* Fractions containing nucleoids (generally fraction #1 at the dense pad) are layered on a preformed 10 mL 20–40 % Optiprep gradient in 1× lysis buffer in an SW41 tube. Samples are centrifuged overnight (14–16 h) at 22,000 rpm (60,000 × *g*). Fractions of 700 μL are collected from the tube bottom. Nucleoids are generally in fraction #5, but this can be confirmed by SYBR Green I staining as above.

4 Notes

1. The small-scale protocol is generally comparable to some commercial kits for mitochondrial isolation, but is much more economical and allows for greater control over the ratio of detergent to the number of cells.
2. The amount of digitonin added is critical since an excess amount will solubilize the mitochondrial outer membrane. It is recommended to start with a conservative amount as indicated and adjust the volume added in steps. After a batch of reagent is adequately tested with a given cell line, it is often possible to target the appropriate amount on the first addition. The amount of digitonin required for permeabilization will depend on the total number of cells being processed due to its stoichiometric binding to membranes.
3. The suggested tube permits the 3 mL syringe to reach the bottom. It is essential that all of the solution is passed through the syringe needle at each stroke.
4. A higher centrifugal force is used when pelleting cells from the higher density mannitol-sucrose solution.
5. Use of a 40× objective is advised to distinguish cells from nuclei. The nuclei of lysed cells appear as rounded structures, generally with clean margins, whereas nuclei of intact cells will be surrounded by other organelles (endoplasmic reticulum, Golgi apparatus, and mitochondria) and the plasma membrane. If necessary, increase the number of passages through the syringe to increase the percentage of cell lysis. Cell lines differ in the extent of association of other organelles, especially endoplasmic reticulum, to nuclei.
6. If nuclei can still be seen in the second post-nuclear supernatant, a third centrifugation will help reduce contamination.

Also note that swinging bucket rotors are preferred when available to avoid streaking of pelleted material along the wall of a fixed angle centrifuge.

7. It is important to AVOID exposing the cell lysate to high salt until after the nuclei have been removed by centrifugation to limit the tendency of organelles to aggregate and to avoid salt extraction of non-histone nuclear proteins.
8. When alternative combinations of centrifuge and rotor are used, alter the angular velocity to conserve the centrifugal force. Conversion calculators are available on-line, such as https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/centrifugation/rotors/index.htm?t=3.
9. The mitochondria may form a layer that can impede the sedimentation of other dense organelles, so that overloading is undesirable. The mitochondrial layer may also have some tendency to adhere to the wall of the centrifuge tube, requiring an effort to dislodge it. We prefer to use a sterile Pasteur pipette to collect this layer.
10. We have found the sucrose gradient and one re-pelleting step is sufficient to physically remove nucleases. If there is evidence of degradation of mitochondrial nucleic acids, an additional resuspension in MSH and sedimentation may be performed.
11. The continuous exposure to non-ionic detergent during sedimentation assures that membrane proteins are removed.
12. We generally number fractions from the bottom. Nucleoids sediment to the pad in fraction 1. This is an imprecise fractionation, but this is a preparative procedure intended to separate free ribosomes from nucleoids. These conditions do not optimally separate large and small ribosomal subunits, for example.
13. SYBR Green I and II are marketed as selective for DNA and RNA, respectively, but both dyes have some ability to detect both forms of nucleic acid.

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Chapter 7

Tracking Mitochondrial DNA *In Situ*

Anna Ligasová and Karel Koberna

Abstract

The methods of the detection of (1) non-labeled and (2) BrdU-labeled mitochondrial DNA (mtDNA) are described. They are based on the production of singlet oxygen by monovalent copper ions and the subsequent induction of DNA gaps. The ends of interrupted DNA serve as origins for the labeling of mtDNA by DNA polymerase I or they are utilized by exonuclease that degrades DNA strands, unmasking BrdU in BrdU-labeled DNA. Both methods are sensitive approaches without the need of additional enhancement of the signal or the use of highly sensitive optical systems.

Key words Mitochondrial DNA, Mitochondrial DNA replication, Copper ions, Biotin-16-dUTP, Singlet oxygen

1 Introduction

The visualization of mitochondrial DNA (mtDNA) can be performed using several approaches including DNA-specific dyes. A typical example represents DAPI (4′,6-diamidino-2-phenylindole). It is also a very illustrative example demonstrating some problems with the detection of mtDNA. Although DAPI is a very common stain of nuclear DNA, its application for the detection of mtDNA has provided controversial results. Some previous studies have even claimed that DAPI stains only the mtDNA of unicellular organisms such as trypanosomes and Schistosoma mansoni, yeasts, and plant cells [1]. These organisms have relatively large mtDNA (50–200 kb), whereas most animal cells have a small mtDNA (<20 kb) [1]. Although DAPI can be used for the detection of mammalian DNA as well, its application usually requires the use of sensitive cameras with an adequate dynamic range and can be obscured by the presence of mitochondrial RNA [1, 2]. Other dyes that can be used for staining mtDNA in fixed or living cells are PicoGreen [3–5], SYTO13 [6], ethidium bromide [7, 8], or SYBR Green I [9]. Also in this case highly sensitive systems should be used to detect mtDNA. Other approaches for the detection of mtDNA are based

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on the use of antibodies against double-stranded DNA [7] or *in situ* hybridization [9, 10]. A very specific approach of mtDNA visualization is the use of fluorescent proteins, such as GFP tagging of the mitochondrial core nucleoid factor Twinkle [2]. Another approach is based on the incorporation of 5-bromo-2′-deoxyuridine (BrdU) or 5-ethynyl-2′-deoxyuridine (EdU) followed by their detection using anti-BrdU antibodies or click chemistry, respectively (e.g. [8, 11–13]). Although this approach reveals only the mtDNA with incorporated BrdU or EdU, it can provide important data about the dynamics of the replication of mtDNA. As longer labeling pulses are normally used, BrdU is preferred to EdU, as EdU is a highly toxic nucleoside analog with a detrimental effect on cell growth [14–19]. On the other hand, BrdU revelation requires additional treatment. It typically includes treatment with hydrochloric acid [12, 20]. Such treatment however results in changes of the cell structure and destruction of DNA, RNA, and proteins [21].

The methods of *in situ* detection of non-labeled (1) and BrdU-labeled (2) mtDNA are described here. They are based on the DNA scission by a radical-oxidative reaction on the carbon atoms of the deoxyribose followed by a set of elimination reactions including the elimination of the nucleobases, finally resulting in the cleavage of the internucleotide linkages and the formation of DNA gaps. In the first method, the ends of interrupted DNA are utilized as the origins, enabling the synthesis of labeled DNA strands of mtDNA. In the second method, the DNA ends are utilized by exonuclease that degrades DNA strands, unmasking BrdU in the newly replicated DNA. Both methods represent very sensitive approaches that usually do not disturb the localization of other cellular components.

2 Materials

2.1 Solutions and Apparatus

1. 10× PBS (phosphate buffered saline; stock solution): 1.370 M NaCl, 30 mM KCl, 160 mM Na_2HPO_4, and 20 mM KH_2PO_4. Adjust the pH to 7.4 with 10 N sodium hydroxide, add deionized water to the final volume. Sterilize in an autoclave. Store at room temperature (RT); after opening, store the bottle in a refrigerator. To prepare the working solution of PBS (1× PBS), dilute ten times the stock solution of PBS in deionized water. Store at 4 °C.
2. 2 % (w/v) Formaldehyde in 1× PBS buffer, pH 7.4 (working solution): Mix the paraformaldehyde with deionized water (one-half of the final fixative volume) in an Erlenmeyer flask. Heat to 60 °C on a stir plate. When moisture forms on the sides of the flask, add sodium hydroxide (~8 drops of 1 N solution per 0.5 L), and stir until the solution clears. Cool the solution

under the faucet. Adjust the pH if necessary and fill with deionized water to the final volume. Store at –20 °C.

Formaldehyde is toxic. Use gloves, prepare and use fixatives under a fume hood.

3. 0.2 % (v/v) Triton X-100 in 1× PBS buffer, pH 7.4 (working solution): Heat 1× PBS to ~40 °C. Add 100 % Triton X-100. Mix for 5 min on a vortex and cool to RT (*see* **Note 1**). Do not store.
4. 1 M Tris–HCl, pH 8.0 (stock solution): Dissolve Tris base in deionized water. Add concentrated hydrochloric acid to adjust the pH to 8.0 and fill with deionized water to the final volume. Store at 4 °C (*see* **Note 2**).
5. 1 M Tris–HCl, pH 7.5 (stock solution): Dilute Tris base in deionized water. Add concentrated hydrochloric acid to adjust the pH to 7.5 and fill with deionized water to the final volume. Store at 4 °C (*see* **Note 2**).
6. 100 mM Tris–HCl, pH 7.5 (working solution): Dilute ten times the stock solution of Tris–HCl in deionized water. Store at 4 °C.
7. Mounting solution for glass slides (working solution): 90 % (v/v) glycerol and 50 mM Tris–HCl, pH 8.0. Add 1,4-diazabicyclo[2.2.2]octane to the final concentration of 2.5 % (w/v) to the solution. Store at –20 °C.
8. 1 M Hepes, pH 7.4 (stock solution): Dissolve Hepes in deionized water (4/5 of the final volume). Adjust the pH with 10 N NaOH. Fill with deionized water to the final volume. Store at 4 °C.
9. 1 M NaCl (stock solution): Dilute sodium chloride in deionized water. Store at 4 °C.
10. 100 mM Tris–HCl, pH 7.5 with 100 mM NaCl (working solution): Dilute ten times the stock solution of Tris–HCl and NaCl in deionized water. Store at 4 °C.
11. Cleavage solution A (stock solution): 20 mM copper(II) sulfate and 500 mM sodium chloride. Dissolve copper(II) sulfate in deionized water, add 1 M NaCl (stock solution) and fill with deionized water to the final volume. Store at 4 °C.
12. Cleavage solution B (stock solution): 50 mM sodium ascorbate and 200 mM Hepes pH 7.0. Dissolve sodium ascorbate in deionized water, add 1 M Hepes (stock solution) and fill with deionized water to the final volume. As aqueous solutions of sodium ascorbate are subject to quick air oxidation, prepare aliquots in Eppendorf tubes, seal them with parafilm and store at –20 °C.

13. 1 mg/mL poly-D-lysine for coating of coverslips (stock solution): Dissolve poly-D-lysine in deionized water. Store aliquots at –20 °C.
14. 1 % (w/v) Gelatin: Dissolve gelatin in heated deionized water (temperature should not exceed 45 °C). Store aliquots at 4 °C.
15. Shaker or similar apparatus for mixing samples with the temperature regulation (e.g. Thermomixer Comfort, Eppendorf) with adaptors for well-plates and 50 mL Falcon Tubes.
16. Vortex for quick mixing of solutions.

2.2 Detection of Non-labeled Mitochondrial DNA

1. Working cleavage solution: Mix one volume of the cleavage solution A, one volume of the cleavage solution B, and twenty-three volumes of deionized water just prior to the DNA cleavage.
2. Solution for enzymatic labeling: DNA polymerase I (final concentration: 0.2 U/μL), a buffer for DNA polymerase I (final concentration: 1×), mixture of 0.05 mM dATP, dGTP, dCTP and alternatively biotin-16-2′-deoxyuridine-5′-triphosphate (biotin-dUTP) or digoxigenin-11-2′-deoxyuridine-5′-triphosphate (digoxigenin-dUTP) or Alexa Fluor® 555-aha-2′-deoxyuridine-5′-triphosphate (Alexa Fluor 555-dUTP; *see* **Notes 3** and **4**).
3. Antibodies: anti-biotin antibody or anti-digoxigenin antibody (primary antibodies), antibodies against anti-biotin or anti-digoxigenin antibody conjugated with fluorescent labels (secondary antibodies). Dilute the primary and secondary antibodies in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl. Use the dilution recommended by the manufacturer of the antibodies.

2.3 Detection of BrdU-Labeled Mitochondrial DNA

1 10 mM BrdU (stock solution): Dilute BrdU in deionized water and adjust the pH to 7.4. Store at –20 °C.

2 Working cleavage solution: Mix one volume of the cleavage solution A, one volume of the cleavage solution B, and three volumes of deionized water just prior the DNA cleavage.

3 Primary antibody solution: anti-BrdU antibody (*see* **Note 5**), exonuclease III (final concentration: 1 U/μL, *see* **Note 6**) and buffer for exonuclease III (final concentration: 1×). Use the dilution of the antibody recommended by the manufacturer.

4 Secondary antibody: Antibody against anti-BrdU antibody conjugated with fluorescent labels. Dilute the secondary antibody in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl. Use the dilution recommended by the manufacturer of the antibodies.

3 Methods

In the case of the detection of non-labeled mtDNA, proceed directly according to the part From Fixation to DNA Cleavage. In the case of the detection of BrdU-labeled mtDNA, incubate your samples first with 10 μM BrdU (final concentration) for the required time at 37 °C in a humidified atmosphere containing 5 % CO_2, or in appropriate conditions depending on the material used before the fixation step, and then proceed according to the part From Fixation to DNA Cleavage as well. The protocol is adopted for cells grown on coverslips or in well dishes with glass bottoms. In the case of cells grown in suspension, first attach the cells to glass coverslips (for tissues, *see* **Note 7**). This quick and easy protocol can be used for the attachment of the cells: put the drop of the cell suspension on the coverslip (50 μL per rounded coverslip with a diameter of 13 mm) coated with poly-D-lysine (*see* **Note 8**), leave for 2 min, remove the excess of the medium using filter paper (put the paper gently to the edge of the coverslip and wait until the excess of the medium is removed) and continue with the part From Fixation to DNA Cleavage.

3.1 From Fixation to DNA Cleavage

All steps are performed at room temperature if not stated otherwise.

1. Fix samples with 2 % formaldehyde in 1× PBS for 10 min.
2. Wash three times in 1× PBS.
3. Permeabilize the samples in 0.2 % Triton X-100 in 1× PBS for 10 min.
4. Wash three times in 1× PBS.
5. Adjust the temperature of the deionized water and stock cleavage solution A to 20 °C.
6. Prepare a fresh working cleavage mixture as described in the Materials, parts Detection of Non-labeled Mitochondrial DNA or Detection of BrdU-labeled Mitochondrial DNA (*see* **Notes 9–12**).
7. Mix on a vortex for 5 s.
8. Remove the buffer from the samples and immediately add the prepared working cleavage mixture.
9. Put the samples on the shaker for 10 s, 300 rpm, 20 °C in the case of the non-labeled mtDNA labeling or for 60 s, 300 rpm, 20 °C in the case of BrdU-labeled mtDNA labeling.
10. Remove the working cleavage mixture, add 100 mM Tris–HCl, pH 7.5 to the samples and wash three times in this buffer.

11. Place the coverslips on their backs on the filter paper and immediately without drying the cells place them on the droplets (100 μL) of 100 mM Tris–HCl, pH 7.5.

3.2 Detection of Non-labeled Mitochondrial DNA

Incubate the coverslips on 100 μL droplets of solutions, or use 200 μL of solutions per well in the case of 96-well dishes if not stated otherwise.

1. Incubate the samples for 20 min in the solution for enzymatic labeling. Use 20 μL per coverslip or 50 μL per well in the case of 96-well plates.
2. Wash the samples three times in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl.
3. If using Alexa Fluor 555-dUTP, proceed according to step 8.
4. If biotin-dUTP or digoxigenin-dUTP are used for the labeling, incubate the samples with the prepared primary antibody for at least 30 min at RT. Use 20 μL per coverslip or 50 μL per well in the case of 96-well plates.
5. Wash the samples four times in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl.
6. Incubate the samples with the prepared secondary antibody for at least 30 min, RT. Use 20 μL per coverslip or 50 μL per well in the case of 96-well plates.
7. Wash the samples four times in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl.
8. In the case of coverslips, remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on filter paper.
9. Apply drops of the mounting medium on the glass slide (3–5 μL) and carefully lower the coverslips onto the mounting medium to avoid trapping any air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 μL of the mounting media.
10. Use nail polish to seal the coverslips and protect the medium from drying. Well dishes and coverslips can be stored at −20 °C. In the case of well dishes, seal them with parafilm to protect the medium from drying on them.

    An example of the visualization of non-labeled mtDNA is shown in Fig. 1.

3.3 Detection of BrdU-Labeled Mitochondrial DNA

Incubate the coverslips on 100 μL droplets of solutions, or use 200 μL of solutions per well in the case of 96-well dishes if not stated otherwise.

1. Incubate the samples with the primary antibody solution for at least 30 min. Use 20 μL per coverslip or 50 μL per well in the case of 96-well plates.

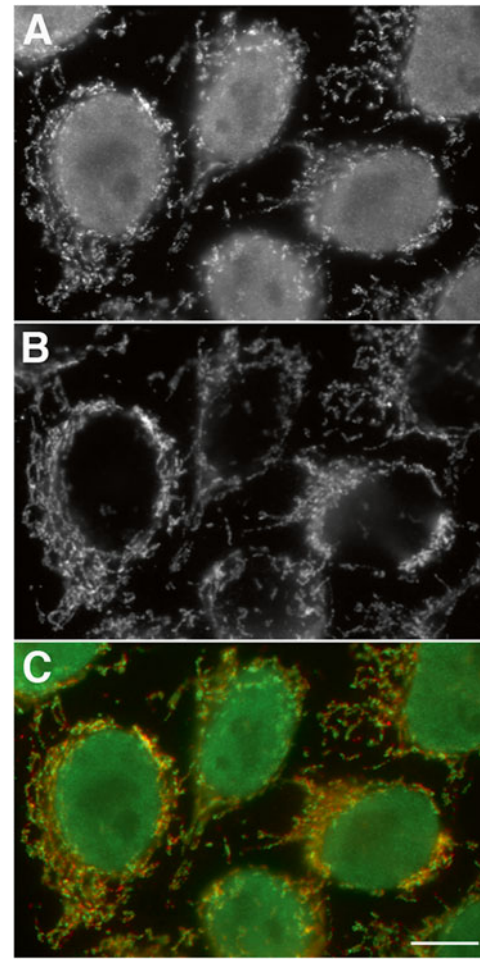

Fig. 1 Detection of non-labeled mitochondrial DNA. The detection of mitochondrial DNA by a 10-s cleavage in a solution containing copper(I) ions followed by the labeling of mitochondrial DNA by DNA polymerase I and biotin-dUTP (**a**, **c**; *green* in the color image) is shown. The cleavage solution consisted of 0.8 mM $CuSO_4$, 100 mM NaCl, 2 mM sodium ascorbate, and 40 mM Hepes. The anti-mitochondrial $MTCO_2$ antibody (**b**, **c**; *red* in the color images) was used for the identification of mitochondria. Bar: 10 μm

2. Wash the samples four times in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl.
3. Incubate the samples with the prepared secondary antibody for at least 30 min at RT. Use 20 μL per coverslip or 50 μL per well in the case of 96-well plates.
4. Wash the samples four times in 100 mM Tris–HCl, pH 7.5 and 100 mM NaCl.
5. In the case of coverslips, remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on filter paper.

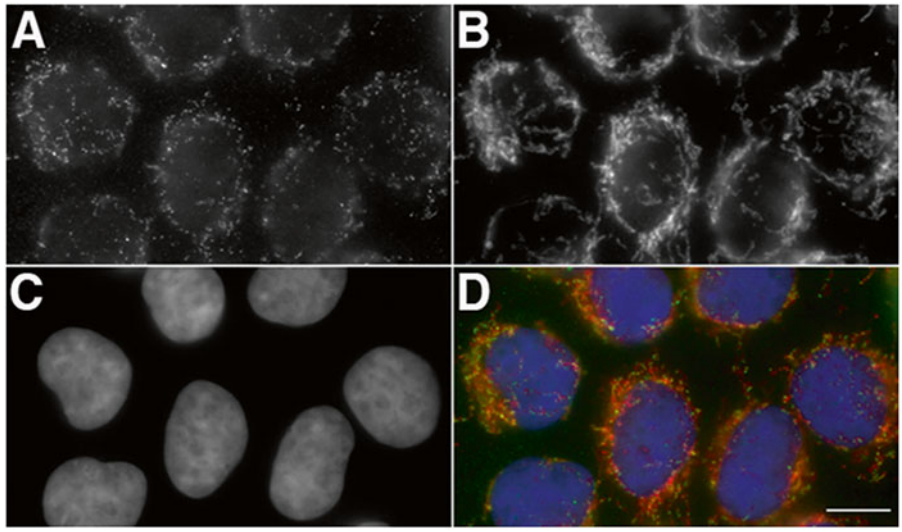

Fig. 2 Visualization of BrdU-labeled DNA. The detection of the BrdU-labeled DNA (**a**, **d**; *green* in the color image) after a 2 h BrdU pulse in cells treated with copper(I) for 60 s followed by exonuclease III cleavage is shown. The cleavage solution consisted of 4 mM $CuSO_4$, 100 mM NaCl, 10 mM sodium ascorbate, and 200 mM Hepes. BrdU-labeled mtDNA was stained using chicken polyclonal anti-BrdU antibody. A simultaneous colocalization with the mitochondrial marker $MTCO_2$ has been performed (**b**, **d**; *red* in the color image). DNA was stained by DAPI (**c**, **d**; *blue* in the color image). Bar: 10 μm

6. Apply drops of the mounting medium on the glass slide (3–5 μL) and carefully lower the coverslips onto the mounting medium to avoid trapping any air bubbles. In the case of well dishes, aspirate the buffer and add 100 μL of mounting media.
7. Use nail polish to seal the coverslips and protect the medium from drying. Well dishes and coverslips can be stored at −20 °C. In the case of well dishes, seal them with parafilm to protect the medium from drying on them.

   An example of the detection of BrdU-labeled mtDNA is shown in Fig. 2.

4 Notes

1. Triton X-100 is a viscous liquid. The use of a pipette for viscous liquid pipetting is recommended. Alternatively, cut off the end of the tip and use the classic pipette.
2. The pH values of Tris buffer solutions are temperature and concentration-dependent. Between 5 and 25 °C, the pH value increases an average of 0.03 pH units for each °C decrease in temperature. As the buffer temperature increases from 25 to 37 °C, the pH value decreases an average of 0.025 pH units per °C. Increasing the concentration of Tris from 0.05 to

0.5 M will increase the pH value by about 0.05 units. Decreasing the concentration from 0.05 to 0.005 M will decrease the pH value by about 0.05 units. As the effect of the temperature is relatively high and the temperature of the Tris buffer increases during the addition of HCl, allow the solution to cool to RT before making the final adjustments of the pH.

3. When biotin-dUTP and digoxigenin-dUTP are used, add 0.05 mM dTTP to the solution for enzymatic labeling. This usually results in an increase of the signal. Do not add dTTP if Alexa Fluor 555-dUTP is used for the detection of mtDNA. The addition of dTTP can completely eliminate the signal.
4. Besides DNA polymerase I labeling of mtDNA, another possibility is the use of terminal deoxynucleotidyl transferase TdT [22]. Although TdT can theoretically provide a higher signal than DNA polymerase I, as TdT activity does not require DNA matrices, its use requires a pre-incubation step with alkaline phosphomonoesterase (e.g. shrimp alkaline phosphomonoesterase, SAP) in order to reconstitute the hydroxyl groups at the 3′ ends of the gaps (Fig. 3, [23]).
5. We tested several antibodies raised against BrdU. We received the best results with the monoclonal antibody clone B44 (Becton Dickinson) and chicken polyclonal anti-BrdU antibody (Abcam). According to our results, the choice of a primary antibody is the crucial prerequisite for the successful detection of BrdU-labeled mtDNA as some antibody clones did not provide any signal or the signal was very low.
6. The presence of exonuclease is absolutely necessary for the detection of BrdU-labeled mtDNA, although the localization of BrdU-labeled nuclear DNA can be performed without the use of exonuclease. It is probably a consequence of a much higher concentration of DNA in the nucleus. Despite that, we recommend the use of exonuclease also in the case of the detection of nuclear DNA as it increases the signal progressively and enables the use of much lower concentrations of copper(I) ions.
7. Although the described methods were primarily developed for cells cultivated in cell cultures, they can also be applied for small pieces of tissues. In that case, prepare tissue spreads. Put the small tissue sample between two coverslips coated with, e.g. poly-D-lysine or 1 % gelatin and spread it over the surface of coverslips by means of the fine pressure of the tweezers on the coverslip. Separate the coverslips and put them in the fixative.
8. To coat the coverslips with poly-D-lysine, prepare a working solution of poly-D-lysine (50 μg/mL). Immediately before use, wash the coverslips in 95 % ethanol and dry them. Place coverslips in the Petri dish with the poly-D-lysine solution in a single layer and incubate for 1 h at 37 °C. Remove the coverslips from the solution and let them dry. Place the coverslips on

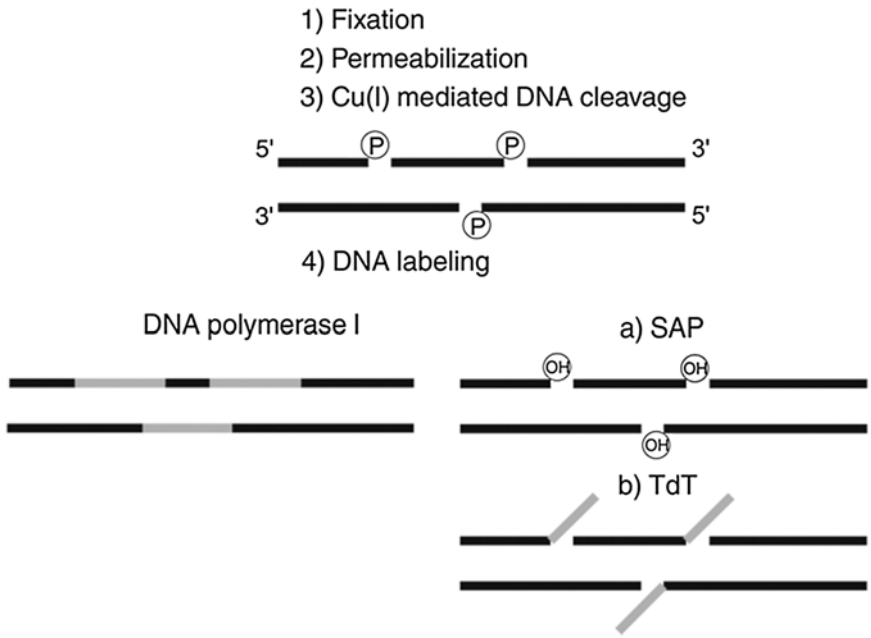

Fig. 3 Scheme of the protocol. A simplified scheme of two basic alternatives of labeling cellular double-stranded DNA is shown. The common steps (fixation, permeabilization, and copper(I)-mediated DNA cleavage leading to gap formation) are followed by the labeling of DNA by means of DNA polymerase I or TdT. In the case of TdT, it is necessary to use the pre-incubation step with SAP in order to reconstitute the hydroxyl groups at the 3′ ends of the gaps. P and OH designate the phosphate and hydroxyl groups at the 3′ ends of the gaps, respectively (from: Ligasová et al., PLoS ONE 2012, 7(12): e52584)

filter paper with the coated side upwards as they can stick to the surface otherwise.

9. DNA cleavage using copper ions is a critical step. The extent of the cleavage has to be controlled as high DNA cleavage can result in the loss of DNA, proteins bound to DNA or even to the loss of other cellular components. On the other hand, low cleavage does not provide a sufficient amount of gaps. The cleavage intensity can be monitored, e.g. by DAPI staining of cell nuclei. A substantial decrease of DAPI signal clearly indicates the progressive cleavage of DNA.
10. The cleavage is influenced by several factors. The cleavage is directly proportional to the temperature, incubation time, copper(I) ions, and oxygen concentration.

    If DNA cleavage is high (a substantial decrease of DAPI signal is observed) or low (no or low mtDNA- or BrdU-derived signal is observed), it is necessary to adjust at least some of these parameters. We recommend to change the concentration of the copper(II) sulfate and the concentration of sodium ascorbate first. The concentration of the copper(II)

sulfate, sodium ascorbate, and Hepes buffer should be changed proportionally. The ratio between the concentration of the copper(II) sulfate, sodium ascorbate, and Hepes buffer should be 1:2.5:50. The effect of NaCl on cleavage is relatively low; however, it should not be substantially above 100 mM in the final mixture.

11. The concentration of oxygen is a critical parameter. Usually, it is necessary to aerate the cleavage solution by mixing. On the other hand, intensive mixing prior to the application of the cleavage solution can result in the complete inactivation of the cleavage mixture. Aeration by mixing during incubation is especially important in the case of the detection of BrdU-labeled mtDNA. It usually requires a higher number of gaps than the detection of non-labeled mtDNA. In practice, we recommend using 0.25 mL of the final working cleavage mixture per 1 cm^2 of cultivation area. It provides a sufficient amount of the mixture and at the same time it can be efficiently aerated by shaking on a laboratory shaker.
12. The necessity of more extensive cleavage for the detection of BrdU-labeled mtDNA as compared to non-labeled mtDNA can result from the fact that any gap can provide a signal during DNA polymerase I-mediated labeling. On the other hand, only some gaps are close enough to incorporated BrdU that has to be revealed by the exonuclease degradation of the opposite strand of DNA.

Acknowledgement

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Part III

Analysis of Mitochondrial DNA Replication, Mitochondrial DNA-Encoded Protein Translation and Mitochondrial DNA Copy Number

Chapter 8

Analysis of Replicating Mitochondrial DNA by *In Organello* Labeling and Two-Dimensional Agarose Gel Electrophoresis

Ian J. Holt, Lawrence Kazak, Aurelio Reyes, and Stuart R. Wood

Abstract

Our understanding of the mechanisms of DNA replication in a broad range of organisms and viruses has benefited from the application of two-dimensional agarose gel electrophoresis (2D-AGE). The method resolves DNA molecules on the basis of size and shape and is technically straightforward. 2D-AGE sparked controversy in the field of mitochondria when it revealed replicating molecules with lengthy tracts of RNA, a phenomenon never before reported in nature. More recently, radioisotope labeling of the DNA in the mitochondria has been coupled with 2D-AGE. In its first application, this procedure helped to delineate the "bootlace mechanism of mitochondrial DNA replication," in which processed mitochondrial transcripts are hybridized to the lagging strand template at the replication fork as the leading DNA strand is synthesized. This chapter provides details of the method, how it has been applied to date and concludes with some potential future applications of the technique.

Key words DNA replication, DNA labeling, Mitochondrial DNA, Two-dimensional agarose gel electrophoresis, 2D-AGE, Replication intermediates, Humans, Mammals

1 Introduction

The DNA in mitochondria is geared to the production of thirteen polypeptides that are essential for aerobic respiration and ATP production. Mammalian mitochondrial DNA is typically arranged as closed, circular molecules of 16 kb pairs. Mitochondrial DNA (mtDNA) is reliant on a host of nuclear-encoded proteins for its maintenance and expression. A plethora of mutations of mtDNA are known causes of human disease, and mutations in the nuclear genes required for its replication can be equally devastating [1–5]. Furthermore mutant mtDNA has been linked to neurodegenerative disorders, in particular Parkinson's disease, and to the normal aging process [6–11]. Hence, greater understanding of the process of mtDNA replication is of considerable importance for many

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aspects of human health and longevity. The technique of two-dimensional agarose gel electrophoresis (2D-AGE) has underpinned recent advances in our understanding of the process of DNA replication in mitochondria and combining it with direct DNA labeling expands its range of applications.

1.1 Neutral Two-Dimensional Agarose Gel Electrophoresis

The development of a method of separating branched molecules based on a combination of structure and mass transformed the study of DNA replication [12–15]. Neutral 2D-AGE has been used to map origins of replication [13, 15–18], identify replication pause sites and termini [14, 19, 20], determine the direction of replication fork movement [19, 21, 22], and define multiple mechanisms of replication [23–28]. The technique is widely applicable, having been used to study DNA replication in eukaryotes, prokaryotes, and viruses [13, 16, 23, 26, 29–35].

The essentials of 2D-AGE are the same irrespective of the source of the DNA. The DNA is usually restriction digested in order to focus on a particular region of the genome of interest, e.g. one suspected to contain a replication origin or pause site. The DNA fragments are first separated in a low percentage agarose gel, at low field strength; in the second dimension, a higher field strength and a higher percentage agarose gel impregnated with the DNA intercalating dye, ethidium bromide, are employed (*see* Fig. 1 and the step-by-step protocol). It is in the second dimension that the shape of the DNA molecule (specifically its hydrodynamic volume) becomes a key factor: replication intermediates (RIs) migrate slower than linear molecules of the same mass. Hence, the passage of a replication fork through a fragment gives rise to a series of intermediates of different mobility, which form a characteristic fork, or Y, arc (Fig. 2a). If a fragment contains an origin of replication, a bubble structure will form, which is more retarded in the second dimension than a replication fork. When a replication fork exits a fragment the bubble is cleaved by the restriction enzyme (assuming the nascent strands are duplex DNA (see below)), and so bubble arcs end abruptly (Fig. 2b). The extent of a bubble arc depends on the position of the origin within the fragment; however, a single fragment cannot define the mode of replication. Hence, it is necessary to analyze a series of overlapping fragments to map origins of replication and determine if replication is unidirectional or bidirectional (Fig. 2c). Such an analysis revealed weak bubble arcs associated with multiple fragments of mammalian and avian mtDNA demonstrating the existence of a broad replication initiation zone in vertebrate mtDNA [22, 36].

Ribonucleotide substitution and RNA incorporation are prominent and highly unusual features of mtDNA metabolism. The mtDNA of mammalian cells and tissues contains sporadic ribonucleotides [37, 38] and lengthy tracts of RNA (transcripts, termed bootlaces) are hybridized to the lagging strand template

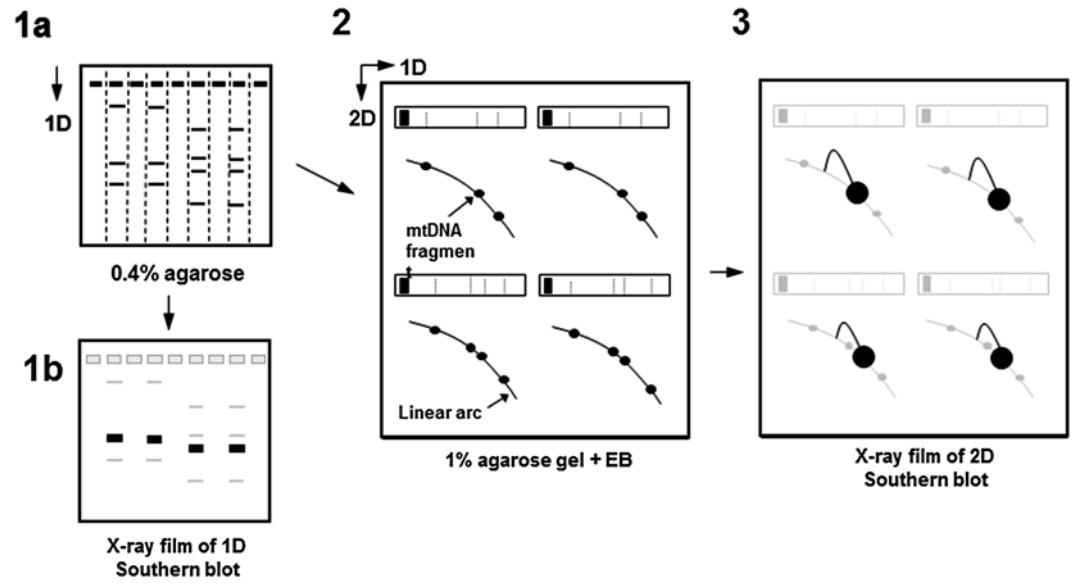

Fig. 1 Schematic diagram of neutral 2D-AGE. Panel 1a depicts a gel after first dimension electrophoresis, *filled black boxes* represent wells of the gel, *narrow horizontal lines* are linear fragments of double-stranded DNA, and *dotted vertical lines* demarcate the lanes to be excised. In a conventional 1D Southern blot (panel **1b**) the lanes are not excised and specific fragments of DNA are detected after transfer to solid support by hybridization to radiolabeled nucleic acid probes. Panel **2**, for 2D-AGE each 1D gel slice is rotated through 90° and a second gel cast around it, four 1D gel slices are shown on a single 2D gel. After second-dimension electrophoresis the linear double-stranded fragments (*black* circles) resolve on a defined arc (*narrow unbroken line*); the position of each fragment after the first dimension separation is shown by a broken faint line in the original 1D gel slice. After blotting and hybridization to detect a specific fragment of (mt)DNA, one of the 1n spots features prominently and its accompanying replication intermediates become visible (panel **3**)

during replication [38, 39]. Because RNA is much more sensitive to extremes of pH than DNA, and the RNA/DNA hybrids are prone to enzymatic degradation during nucleic acid isolation, care is required in the preparation of samples for the study of mtDNA replication. All reagents should be tested for contaminating RNase H activity [40] and sucrose step-gradient purification of the mitochondria is often beneficial. Although in recent years we have produced largely intact RIs from mitochondria of solid tissues prepared solely by differential centrifugation, or even fresh homogenates [41], we are still working toward preparations in which the RNA hybridized to mtDNA is fully intact. This is proving particularly challenging in the case of mtDNA of cultured cells, where even interstrand cross-linking of the mtDNA in *intact cells* is insufficient to preserve fully the bootlaces [41], and this can explain the recently reported distribution of mtSSB bound to mtDNA in HeLa cell mitochondria [42]. Alternatively, the bootlace mechanism is much sloppier in cultured (aneuploid) cells than *in vivo*, and missing bootlaces are substituted by mtSSB; but in view of the clear threat to mtDNA integrity posed by extensive single-stranded

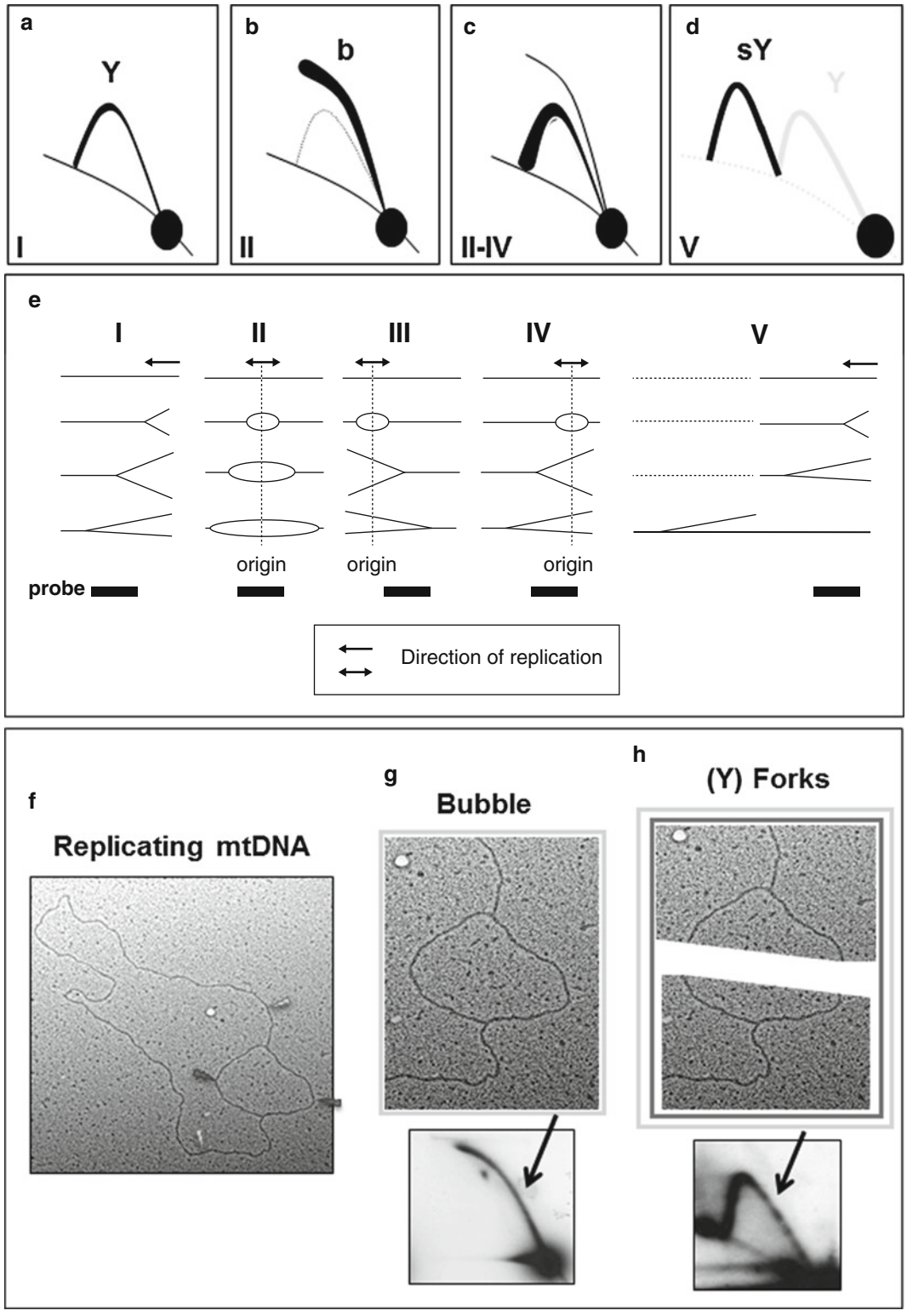

Fig. 2 Interpretations of patterns of replication intermediates fractionated by 2D-AGE. Panels (**a**–**d**): schematic patterns of RIs on 2D-AGE. Panel (**e**), line drawings of structures of RIs. Panel (**a**), a simple replication fork or Y-arc of duplex DNA, replication initiated outside the fragment, interpreted in **e**-I. Panel (**b**), initiation of bidirectional replication from a discrete origin located at the center of a fragment giving rise to a complete bubble arc,

regions formed during replication [43], we view this as highly unlikely even for cancer cells. Ultimately, quantitative methods that measure the coverage of RNA versus mtSSB on actively replicating molecules will be needed in the future to determine the contributions of each to lagging strand template protection.

Although neutral 2D-AGE is not the only means of dissecting DNA replication, it is one of the few methods appropriate to the study of replicating DNA with a high ribonucleotide content. The alternative form of two-dimensional agarose gel electrophoresis that employs alkali in the second dimension (Neutral/alkaline 2D-AGE) [44] is inapplicable to mammalian mtDNA, because it will result in the fragmentation of the template and nascent DNA strands, as well as complete loss of any RNA/DNA hybrid. In our hands, atomic force microscopy is considerably harsher than 2D-AGE, often leading to fragmentation of mitochondrial replication intermediates (Holt and colleagues, unpublished observations). The formation of RNA/DNA hybrids during mtDNA replication is evident as slow-moving or supra Y arcs (Fig. 2d), as such hybrids are refractive to restriction digestion creating two (or more) contiguous fragments. Once mtDNA samples of high purity have been prepared, RNase H is a valuable tool for modifying the RIs in vitro [38, 39] and incorporated RNA can also be retarded or captured using antibodies specific for RNA/DNA hybrids [40].

1.2 Combined 2D-AGE and In Organelle Labeling of mtDNA

A limitation of Southern blots of 2D-AGE is that it shows the steady-state level of replication intermediates. This becomes particularly problematic where there is evidence of multiple mechanisms of replication operating concurrently. If each makes an equal contribution to replication but the rates of DNA synthesis are different then the strongest signal will be contributed by the slowest mechanism. It was also formally possible that many of the RIs detected by 2D-AGE and microscopy methods were dead end

Fig. 2 (continued) because the two forks exit the fragment simultaneously there is no fork arc (the position of the fork arc is shown as a faint broken line for reference purposes). Panel (**b**) is interpreted in **e**-II; the bubble arc increases in intensity as it reaches its apex, giving it a "clubheaded" appearance, due to compression. Panel (**c**), bidirectional initiation from multiple sites across a zone defined by the fragment; in this case initiation at the center of the fragment is only one of many possibilities, at other initiation sites such as those depicted in **e**-III and **e**-IV one fork exits the fragment well before the other, converting the bubble to a Y structure. Panel (**d**), a supra Y (sY) arc of the type associated specifically with mtDNA replication. These result from failure of the restriction enzymes to cut one branch of a RI after the fork has exited a fragment (as depicted in **e**-V). Panel (**f**), an electron microscope image of a replicating DNA molecule cut at two *light gray* restriction sites that preserves the bubble (panel **g**) and a *dark gray* site located at the center of the bubble (assumed to be a bidirectional origin in this interpretation), cleavage of the bubble converts it to fork structures that contribute to the Y arc(s) (panel **h**). Note that the image in panel (**g**) corresponds to one of the points on the arc illustrated in **e**-II, whereas **h** is equivalent to an **e**-I replication intermediate

products. The solution to this problem is to label the DNA in real-time. DNA labeling *in situ* can be achieved using a variety of radioactive or fluorescent nucleotide analogs, and these methods lend themselves to pulse-chase analysis. The cells or extracts are incubated with the label for a defined time and its fate can be followed over an extended period. Critical questions are: do the RIs incorporate label rapidly and are they "chased" into mature DNA?

It had been shown in 1994 that mtDNA could be labeled "*in organello*" by incubating isolated mitochondria with a radioactive deoxynucleotide triphosphate [45]. We discussed the idea of combining 2D-AGE and *in organello* labeling in the late 1990s, but the standard form of 2D-AGE generated such a wealth of data on mtDNA replication in the 2000s that the plan was repeatedly postponed. When eventually tested, the original *in organello* labeling protocol [45] was found to be robust in our hands and required little in the way of modification. Incubations of rat liver mitochondria with ^{32}P-dATP for as little as 5 min strongly labeled the previously assigned RIs, as evidenced by 2D-AGE analysis (Fig. 3), and as with those molecular species detected in the steady-state the supra-Y arcs and most of the material of the bubble arc were grossly modified by RNase H treatment (Fig. 4) [41]. Moreover, following a five-minute pulse the signal of the mitochondrial RIs declined with time, whereas that of mature mtDNA accumulated during a chase of up to 2 h, thereby establishing a precursor product relationship between the proposed RIs and mature mtDNA [41]. In our hands, labeling of total mtDNA peaks anywhere between 2 and 3 h and declines slowly thereafter. Hence it is not possible to extend reactions indefinitely, at least not without further refinement of the protocol.

Our contention that RNA is incorporated on the lagging-strand during the mtDNA replication cycle [38–40] made a firm prediction that could be addressed directly using the *in organello* labeling system. Radiolabeled RNA precursors should like their dNTP counterparts become incorporated in mitochondrial RIs, but they should not accumulate in the mature mtDNA.[1] RNA can be labeled in mitochondria in much the same way as DNA labeling [46]. ^{32}P-UTP labeling of mitochondria followed by DNA isolation and 2D-AGE revealed a pattern of RIs essentially the same as those detected by DNA labeling [41]. In marked contrast to DNA labeling, incubations of at least an hour with ^{32}P-UTP are

[1] Although mature mammalian mtDNA contains a handful of sporadic ribonucleotides [37, 38], they constitute less than 1 % of the total and so can be ignored in this context.

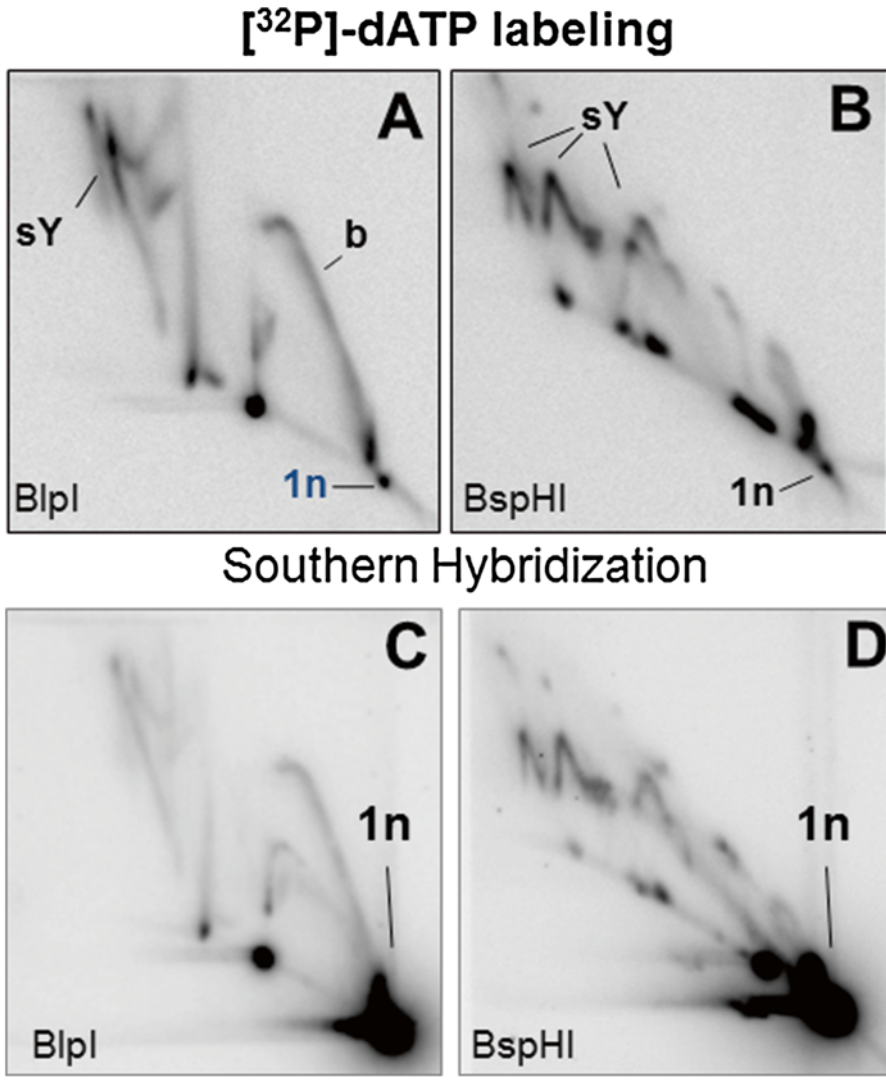

Fig. 3 The majority of mtDNA fragments labeled *in organello* are replication intermediates. After *in organello* labeling of rat liver mitochondria, mtDNA was extracted, digested with BlpI or BspHI and subjected to 2D-AGE. After transfer to solid support, phosphorimager analysis of the BlpI-digested material revealed a prominent bubble arc (b) and a supra-Y (sY) replication fork arc (panels **a** and **b**), substantially the same as those detected by Southern hybridization after probing with a radiolabeled probe (panels **c** and **d**). Note that the probe detects a specific fragment of mature mtDNA of 5 kb (1n), whereas incubation of mitochondria with [α^{32}P]-dATP labels all fragments of the mtDNA during the reaction. The linear fragments of mtDNA cut at all the restriction sites (1n spots) have always been presumed to be non-replicating mtDNA and this is confirmed by the *in organello* labeling method as their contribution to total signal is many fold lower than the steady-state amount indicated by Southern blotting (panels **a** and **b** compared with **c** and **d**)

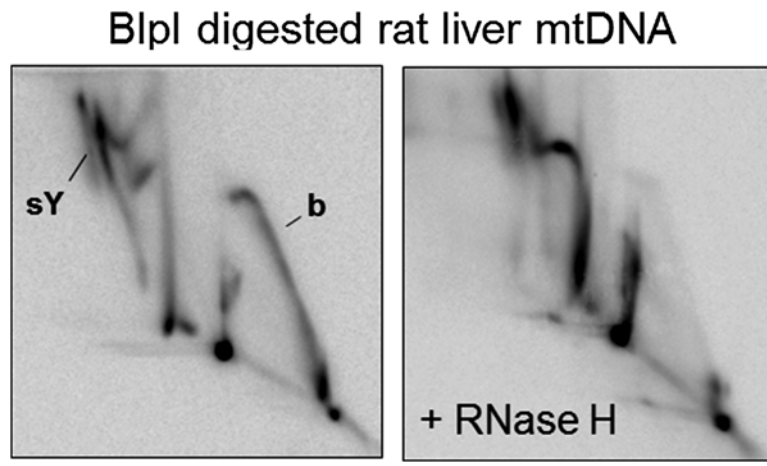

Fig. 4 The majority of mtDNA fragments labeled *in organello* contain extensive RNA/DNA hybrid. *In organello* labeled rat liver mtDNA was digested with BlpI, treated with or without RNase H and the products fractionated by 2D-AGE, transferred to filter-membrane and phosphorimaged. The gross modification of the major arcs (origin-containing bubble (b) arc and supra-Y (sY) arc) indicates that the replication occurring in the mitochondria was predominantly of the bootlace type during the 5 min labeling reaction (see [41])

necessary to be able to detect the mitochondrial RIs,[2] whereas labeling of the pool of free transcripts is evident within a matter of minutes. A DNA chain terminator inhibits *in organelle* labeling, whereas a RNA chain terminator has no impact on replication in isolated mitochondria. The delay in RNA labeling of mtRIs and the ineffectiveness of the RNA chain terminator are both incompatible with RNA synthesis on the lagging-strand concurrent with leading-strand DNA synthesis. Instead, the results suggest preformed RNAs are incorporated at the replication fork, with mitochondrial transcripts being the obvious candidates. As predicted by this interpretation, we found it is possible to recover mitochondrial transcripts from gel-extracted mtRIs [41].

These studies demonstrate the ability of *in organello* labeling to address details of the mechanism of mtDNA replication that cannot be achieved by standard 2D-AGE. However, the first study did reveal a possible limitation. While replication intermediates composed of fully duplex DNA are readily detectable by Southern hybridization [22, 36, 40, 47, 48], they were considerably weaker in the material labeled *in organello* [41]. As described above, this could be taken to imply that coupled leading- and lagging-strand DNA synthesis is slower than the bootlace mechanism of

[2] The milder transfer conditions used for UTP-labeled RIs sometimes prove less efficient than standard alkali blotting but does not account for the difference in signal which is apparent from monitoring of the gels pre-transfer.

replication. However, the RNA incorporation mechanism is itself very slow, so this explanation seems unlikely. More plausibly, organelle isolation entails disruption of the cell, its cytoskeleton and the mitochondrial network, and so this mechanism of replication may not function well outside the cell.

The *in organello* mtDNA replication system provides a testbed to study the role of proteins implicated in mtDNA replication and further details of the mechanism(s). Although it will not be easy to source the material, *in organello* labeling would be ideal to determine if the unusual arrangement of adult heart mtDNA with its innumerable four-way junctions is a replicative, or post-replicative state [49]. Isolated mitochondria retain the capacity to respire and import proteins from the incubation medium and so the levels of individual proteins can be increased during the course of an experiment and the different variants can be assessed in parallel. Recombinant TFAM has already been shown to stimulate 7S DNA synthesis but not increase mtDNA copy number in one study [50]. Testing the DNA helicase Twinkle (C10orf2) is of considerable interest as it increases mtDNA copy number in the mouse, but not in cultured cells [51, 52]. In addition, for replication factors known, or suspected, to be shared between mitochondria and the nucleus, use of the *in organello* system allows physiological effects in mitochondria to be studied specifically, which would be considerably more difficult to accomplish in whole cells. The *in organello* labeling procedure also has the potential to answer many outstanding questions about mtDNA replication in pathological states. For example, by studying the relative rate of incorporation of labeled nucleotide precursors into newly synthesized mtDNA, it should be possible to confirm or refute the idea that pathological Twinkle helicase mutants cause replication stalling [51]. Nucleotide balance is also recognized as a critical parameter influencing mtDNA replication, since imbalances precipitate mtDNA depletion and disease [53–55]. This too may lend itself to analysis *in organello*, although differences in the relative rate of uptake between different nucleotides will need to be taken into account.

2 Materials

2.1 Isolation of Mitochondria from Solid Tissue

1. Phosphate-buffered saline: 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl.
2. Homogenization buffer (HB): 320 mM sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 1 mg/mL essentially fatty acid-free bovine serum albumin (BSA).
3. Motorized Dounce Homogenizer with tight-fitting Teflon pestle.
4. Sprague–Dawley rats approximately 1 month of age.

5. Refrigerated centrifuge with rotor suitable for pelleting material at up to $10{,}000 \times g_{max}$ in volumes of 10–50 mL.
6. Refrigerated micro-centrifuge.

2.2 Protein Estimation

1. UV Spectrophotometer.
2. Sodium dodecyl sulfate (SDS), 0.6 % (w/v).
3. Protein standards.
4. Quartz cuvettes.

2.3 In Organello Labeling

1. Rotisserie-style hybridization oven (*see* **Note 1**).
2. Incubation buffer (IB): 10 mM Tris–HCl, pH 8.0, sucrose and glucose 20 mM each, 65 mM d-sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.5 mM EDTA, 1 mg/mL BSA, 1 mM ADP, 5 mM $MgCl_2$, 5 mM glutamate, and 5 mM malate (*see* **Note 2**).
3. [α-^{32}P]-dATP (3000 Ci/mmol).
4. 1 mM solutions of dTTP, dGTP, dCTP, and dATP.
5. [α-^{32}P]-UTP (12,000 Ci/mmol).
6. 1 mM solutions of ATP, CTP, and GTP.

2.4 Mitochondrial DNA Extraction

1. Lysis buffer (LB): 20 mM HEPES–NaOH, pH 7.8, 75 mM NaCl, 50 mM EDTA.
2. 20 % (w/v) Sodium lauroyl sarcosinate (sarkosyl).
3. Proteinase K (PK): 20 mg/mL in water.
4. PCIA: equilibrated phenol:chloroform:isoamyl alcohol (25:24:1).
5. CIA: chloroform:isoamyl alcohol (24:1).
6. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
7. Isopropanol.
8. Ethanol.

2.5 Nucleic Acid Restriction and Modification, and 2D-AGE

1. Restriction enzymes.
2. RNase T_1, RNase H.
3. 5 M NaCl.
4. 10 mM Tris–HCl, pH 8.0.
5. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
6. 0.5 M EDTA, pH 8.0.
7. 1× TBE: 89 mM Tris base, 87 mM boric acid, 2 mM EDTA, pH 8.0.
8. Loading buffer: 40 % sucrose, 0.025 % (w/v) xylene cyanol & bromophenol blue.
9. Low electroendosmotic (LEEO) agarose & ethidium bromide (EB): 10 mg/mL.

10. Gel tank with ports for buffer circulation, peristaltic pump.
11. Power supply & Ultraviolet (UV) light box.

2.6 Transfer of mtDNA to Solid Support and Hybridization

1. Depurination buffer (DPB): 0.25 N HCl.
2. Denaturing buffer (DNB): 0.5 M NaOH, 1.5 M NaCl.
3. Nylon membrane, 3 MM Whatman filter paper & paper towels.
4. Hybridization buffer: 0.25 M sodium phosphate, pH 7.2, 7 % (w/v) SDS.
5. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
6. Washing buffer 1 (WB1): 1× SSC.
7. Washing buffer 2 (WB2): 1× SSC/0.1 % (w/v) SDS.
8. X-ray film or phosphorimager cassettes.

3 Methods

3.1 Isolation of Mitochondria from Rat Liver

All solutions should be pre-cooled and all operations carried out on ice in a cold room.

1. First excise and discard the gall bladder, then remove the liver from the rat, separate and discard any associated fat, blood, and blood vessels.
2. Weigh the liver. 4 g liver provides enough material for ≥20 labeling reactions.
3. Mince the tissue finely with scissors and rinse repeatedly with ice cold 1:10 diluted HB until there is no residual blood or fat.
4. After the final rinse, add 4 mL HB/g minced liver and homogenize using a motorized tight-fitting Dounce homogenizer until the suspension is smooth (*see* **Note 3**).
5. Centrifuge the homogenate at $1000 \times g_{max}$ for 5 min at 4 °C to pellet nuclei and large debris; repeat this step.
6. Centrifuge the supernatant at $9000 \times g_{max}$ for 5 min at 4 °C to pellet the mitochondria.
7. Discard the supernatant and suspend the mitochondrial pellet in 5 volumes of HB/g; i.e. 20 mL for 4 g of liver (*use a loose fitting glass homogenizer to ensure a homogenous solution*).
8. Divide the solution among 1.5 mL or 2 mL plastic tubes, re-pellet the mitochondria by centrifuging at $10{,}000 \times g_{max}$ for 2 min at 4 °C.
9. Resuspend each pellet in 700 or 800 μL of HB and half the number of tubes; re-pellet the mitochondria by centrifuging at $10{,}000 \times g_{max}$ for 2 min at 4 °C.

10. Resuspend the mitochondrial pellet in IB and leave to equilibrate for the time taken to estimate the protein concentration.
11. Remove 10 μL of mitochondrial suspension and estimate the protein concentration (*see* Subheading 3.2 below).
12. Pellet the mitochondria (10,000 × g_{max} for 2 min at 4 °C) and resuspend the organelles in IB at a concentration of 4 mg/mL protein (*see* **Note 4**).

3.2 Protein Estimation

1. Mix 1 μL of sample with 99 μL 0.6 % SDS in a 0.5 mL tube.
2. Heat to 95 °C for 4 min.
3. Set the spectrophotometer at A_{280nm}, add 100 μL of 0.6 % SDS to two cuvettes and set the reading as zero.
4. Read the $A_{280\ nm}$ of the sample (from **step 1**).
5. Calculate the protein concentration based on 0.21 U of Absorbance = 10 μg/mL. Thus, A280/0.021 = protein concentration × 10 μg/mL (*see* **Note 5**).

3.3 In Organello Labeling of Mitochondrial Nucleic Acid

1. Prepare 1 mL lots of mitochondrial suspension in 2 mL plastic tubes according to the number of reactions required.
2. To each tube add [α-^{32}P]-dATP (3000 Ci/mmol) to a final concentration of 6.6 nM, and cold dTTP, dGTP and dCTP, each 50 μM.
3. Incubate at 37 °C with rotation according to the length of the pulse (5 min to 3 h) (*see* **Note 6**).
4. If a chase is required pellet the mitochondria, 10,000 × g_{max} for 2 min at 4 °C, and resuspend in 1 mL of IB with all four dNTPs (50 μM each). Incubate at 37 °C with rotation according to the length of the chase.
5. For RNA labeling use 6.6 nM [α-^{32}P]-UTP (12,000 Ci/mmol), and 50 μM each of ATP, CTP, GTP, and the four dNTPs (*see* **Note 7**).

3.4 Mitochondrial DNA Extraction

1. After the pulse labeling (and chase) reaction, pellet the mitochondria and resuspend them in 475 μL LB (*without detergent*). Add Proteinase K (stock 20 mg/mL in water) to a final concentration of 0.1 mg/mL and incubate on ice for 30 min.
2. Add 25 μL of 20 % sodium sarkosyl and mix gently.
3. Add an equal volume of PCIA mix gently yet thoroughly.
4. Centrifuge 16,000 × g_{max} for 5 min at 4 °C.
5. Transfer the upper aqueous phase to a clean tube, mix gently with 1 volume of CIA, and spin at 16,000 × g_{max} for 5 min at 4 °C.

6. Recover the upper, aqueous phase and add 1 volume of isopropanol and NaCl to 100 mM, mix gently, and incubate at –20 °C for 1 h or overnight.
7. Centrifuge at 20,000 × g_{max} for 20 min at 4 °C.
8. Wash the pellet with 70 % ethanol and briefly air-dry.
9. Resuspend the pellet in TE, and determine the nucleic acid concentration, e.g. by UV spectrometry.

3.5 Nucleic Acid Modification and 2D-AGE (See Note 8)

1. Digest 3 μg lots of total mitochondrial nucleic acid with 10 U of the appropriate restriction endonuclease(s) for 1–3 h at 37 °C in a total volume of 200 μL.
2. To precipitate the digestion products, add NaCl to 100 mM (maximally 4 μL of 5 M NaCl for a volume of 200 μL), 5 μL Glycogen (10 mg/mL), and 2 volumes of 100 % EtOH (420 μL). Invert the tubes several times. Precipitate at –20 °C overnight or 1 h.
3. Centrifuge the sample at 20,000 × g_{max} for 20 min at 4 °C.
4. Discard the supernatant and wash the pellet with 70 % ethanol.
5. Air-dry the pellet, suspend in TE or in 10 mM Tris–HCl, pH 8.0 if it is to be treated with modifying reagents. If no additional treatment is required then the sample is ready for 2D-AGE.
6. Our standard conditions for the modifying enzymes used to date are 1 U of RNase H for 15 min at 37 °C in 20 mM HEPES–KOH, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT; 100 U of RNase T_1 nuclease in 10 mM Tris–HCl, pH 7.4, 40 mM NaCl, 1 mM $MgCl_2$ for 15 min at 37 °C.
7. Stop the reaction by flash-freezing the sample on dry ice/ethanol.
8. Prepare a 100 mL 0.4 % agarose gel in 1× TBE buffer and cast in a 110 × 140 mm gel tray for 1D-AGE.
9. Submerge gel in a tank containing 1× TBE.
10. Load samples and appropriate size markers (e.g. kilobase ladder).
11. Run the first dimension at 0.7 V/cm for 20 h at room temperature.
12. After 20 h, cut out the lanes containing the samples with the aid of a razor blade and a ruler.
13. Prepare 400 mL of 1 % molten agarose gel in 1× TBE containing 500 ng/mL EB.
14. Rotate each 1D gel slice 90° counter-clockwise and place it in a 200 × 200 mm gel-casting tray (*see* Fig. 1).

15. Remove excess TBE buffer around the gel slice with 3 MM filter paper and adhere the gel slice to the tray with 1 % molten agarose.
16. Cool the 1 % molten agarose to 50 °C in a water bath and add to the gel tray from the opposite side of the gel slice and wait until it has solidified.
17. Place the gel in a tank containing cold 1× TBE with 500 ng/mL EB.
18. Using a peristaltic pump, circulate the buffer from the positive to the negative electrode to avoid the EB accumulating at the cathode.
19. Run the second dimension at 6 V/cm for 6 h at 4 °C.
20. The gel can be dried using a standard gel dryer but the national regulations concerning radioactive condensate must be consulted.

3.6 Transfer to Solid Support (See Note 9)

1. After 2D-AGE, remove the gel from the tank and invert into a glass dish.
2. Add 500 mL DPB for a dish measuring 300 × 400 mm, rock gently for 30 min, and discard solution (*acid turns xylene cyanol green and BPB yellow*).
3. Add 500 mL DNB and rock gently for 5 min. Pour off the solution and repeat the treatment for 25 min (*xylene cyanol and BPB return to their original colors*).
4. Add 500 mL NB and rock gently for 10 min. Discard the solution and repeat the treatment for 10–20 min.
5. Cut a piece of nylon membrane to the dimensions of the gel, wet in water and place face down on the gel removing any bubbles with a roller or glass rod.
6. Soak two sheets of 3 MM Whatman filter paper in water and place them on top of the membrane, again avoiding bubbles.
7. Stack 10–12 cm of paper towel on top of the filter paper.
8. Place a glass plate or a tray on top and a weight to create enough pressure to maintain uniform contact across the gel (*see* **Note 10**).
9. Blot overnight on the bench.
10. Remove and discard paper towels and 3 MM Whatman filter paper. Place the membrane face up on 3 MM Whatman filter paper and let stand for a few minutes.
11. Covalently link the DNA to the membrane in a UV cross-linker: total energy 1200 × 100 mJ/cm^2.
12. Expose the membrane to an X-ray film or phosphorimager cassette for 30 min to several days.

13. Because of the sensitivity of RNA to extremes of pH, DPB and DNB are not appropriate after UTP labeling of mitochondria. Instead the gel should be equilibrated in 6× SSC buffer, 20 mM NaOH for 30 min.
14. Rinse the membrane in 6× SSC, place the gel on a bed of three layers of 3 MM Whatman filter paper soaked in 6× SSC and follow **steps 5–12** above.

3.7 Hybridization to Determine the Steady-State Level of RIs

1. Place the membrane DNA side up in a hybridization tube and add 15 mL pre-warmed hybridization buffer at 65 °C.
2. Incubate in a hybridization oven for at least 30 min at 65 °C.
3. Pour off the solution and repeat the incubation with fresh 15 mL pre-warmed hybridization buffer at 65 °C.
4. Label a mtDNA-specific probe (*see* **Note 11**).
5. Denature the dsDNA probe at 95 °C for 5 min and chill on ice for 2 min.
6. Incubate overnight at 65 °C for dsDNA probes, 60 °C for riboprobes and oligonucleotides >80 mers. Wash temperatures should also be adjusted down for these last two types of probe. In all cases there should be next to no detectable probe in the wash solution at the end of the process.
7. Discard the hybridization solution containing the probe and wash the membrane four times with 50 mL WB1 at 65 °C for 20 min.
8. Wash the membrane twice with 50 mL WB2 at 65 °C for 20 min.
9. Remove the membrane from the tube and air-dry briefly.
10. Wrap the membrane in cling film (Saran Wrap). Expose to X-ray film for 0.5–7 days at –80 °C, or to a phosphor cassette.

4 Notes

1. Any device that enables the mitochondria to be mixed during the labeling reaction should suffice.
2. Incubation buffer can be stored as a 2× stock for at least 6 months at 4 °C.
3. Four to six strokes using a IKA Labortechnik RW 20 motorized homogenizer set at speed 5.
4. 40 mg of enriched mitochondria from 4 g liver is a typical yield providing enough material for 40 reactions.
5. On the first few occasions protein standards should be tested for validation purposes.

6. We adhere the 2 mL tubes to the outside of a glass hybridization tube using a piece of autoclave tape.
7. Mitochondrial RIs are barely detectable with [α-^{32}P]-UTP pulses shorter than 1 h; 2 h is recommended.
8. The conditions described for 2D-AGE are designed to give optimal resolution of 3–4-kb fragments. In the case of fragments of >5 kb, 1D electrophoresis is in a 0.35 % agarose gel at 1.5 V/cm for 20 h, and 2D at 3 V/cm for 18 h in a 0.875 % agarose gel. For fragments <3 kb use 0.9 V/cm for 20 h for 1D and 0.55–1.0 % (w/v) agarose, at room temperature (1D) and 2D 9 h at 260 mA, 1.5–2.0 % agarose, at 4 °C.
9. Although capillary transfer to solid support (Southern blotting) is not required for *in organelle*-labeled mtDNA, it is convenient to transfer the mtDNA fragments to a membrane as drying large radioactive agarose gels is somewhat problematic and subject to strict rules. Moreover, blotting is essential if one wishes to determine the relative abundance of actively replicating molecules compared to the steady-state level of RIs (*see* Subheading 3.7).
10. High pressure does not facilitate capillary transfer, and post-denaturation capillary transfer works well without a reservoir of "transfer" buffer. The efficiency of transfer of *in organelle-labeled* products can be monitored readily by means of a Gieger counter.
11. Random hexamer-labeled dsDNA probes of cloned or PCR amplified mtDNA can be generated with commercial kits or by assembling the individual reagents. End-labeled oligonucleotides and riboprobes are both effective ways of gaining strand-specific information.

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Chapter 9

Translation and Assembly of Radiolabeled Mitochondrial DNA-Encoded Protein Subunits from Cultured Cells and Isolated Mitochondria

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Abstract

In higher eukaryotes, the mitochondrial electron transport chain consists of five multi-subunit membrane complexes responsible for the generation of cellular ATP. Of these, four complexes are under dual genetic control as they contain subunits encoded by both the mitochondrial and nuclear genomes, thereby adding another layer of complexity to the puzzle of respiratory complex biogenesis. These subunits must be synthesized and assembled in a coordinated manner in order to ensure correct biogenesis of different respiratory complexes. Here, we describe techniques to (1) specifically radiolabel proteins encoded by mtDNA to monitor the rate of synthesis using pulse labeling methods, and (2) analyze the stability, assembly, and turnover of subunits using pulse-chase methods in cultured cells and isolated mitochondria.

Key words Mitochondria, Mitochondrial DNA (mtDNA), Oxidative phosphorylation (OXPHOS), Respiratory chain, Complex assembly, Pulse labeling, Pulse-chase labeling, Blue-native PAGE (BN-PAGE)

1 Introduction

Mitochondria are responsible for the majority of cellular ATP production through the activity of five multi-subunit enzyme complexes, comprising the electron transport chain. Of these, four complexes (complex I or NADH-ubiquinone oxidoreductase; complex III or ubiquinol-cytochrome *c* oxidoreductase; complex IV or cytochrome *c* oxidoreductase; and complex V or F_oF_1-ATP synthase) harbor subunits that are encoded by both the nuclear and mitochondrial genomes. Complexes I and II utilize the reducing equivalents NADH and $FADH_2$ as entry points for electrons respectively, while complexes I, III, and IV couple the electron transport to the translocation of protons across the inner membrane, resulting in a membrane potential. The established

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membrane potential is then used by complex V to generate ATP from ADP and inorganic phosphate. The majority of mitochondrial proteins, including components of the electron transport chain, are encoded by nuclear genes and following their translation in the cytosol, are imported into the organelle by specific import machineries [1]. In mammals, mtDNA encodes 13 proteins—all of which are subunits of the respiratory chain complexes, as well as 22 tRNAs and 2 rRNAs required for subunit translation [2]. These proteins are translated within the organelle before they assemble with the imported subunits to form mature respiratory complexes.

Using radiolabeled versions of the sulfur-containing amino acids methionine and cysteine ([^{35}S]-Met/Cys) in the presence of an inhibitor of cytosolic ribosomes, we can specifically label mtDNA-encoded proteins in a cell culture system. Pulse labeling is used to investigate the translation of newly synthesized mtDNA products. This type of experiment is carried out in the presence of the irreversible cytosolic ribosome inhibitor emetine hydrochloride [3]. In order to carry out pulse-chase analysis, the cytosolic inhibitor is required to be reversible as to allow normal protein synthesis to occur following the pulse period. In this case either anisomycin or cycloheximide can be used, as both compounds are inhibitors of the cytosolic ribosome [4, 5]. Treatment with these inhibitors should be for a minimal time as they can be toxic to cells—e.g. anisomycin has been shown to also strongly activate stress kinases and the induction of genes including c-fos, fosB, and JunB [6, 7] while cycloheximide has been shown to induce apoptosis in various cell types [8, 9]. Replacing the labeling media with regular media following the pulse is sufficient to reverse the inhibition of cytosolic ribosomes allowing normal translation of proteins to proceed. This technique, coupled to Blue-native PAGE analysis, has been useful in understanding the assembly of these mtDNA-encoded proteins into mature OXPHOS complexes, as well as understanding defects in respiratory complexes leading to mitochondrial disease [10–16]. To assess *de novo* mitochondrial protein synthesis, pulse labeling can be also employed in freshly isolated mitochondria from animal tissues (*in organello*). Due to the purification of the organelle and hence removal of the "extra"-mitochondrial compartments, the supply of nuclear-encoded subunits is abolished so that a pulse-chase experiment to assess turnover or assembly of subunits into complexes is not possible.

In this chapter, we outline general methods for the radiolabeling of mtDNA-encoded subunits to investigate their synthesis, stability, and turnover. This, together with analysis by Blue-native PAGE, SDS-PAGE, or a combination of both can be applied to investigate respiratory chain biogenesis in many different contexts.

2 Materials

2.1 Pulse and Pulse-Chase Radiolabeling in Cultured Cells

1. Cultured cell lines to be radiolabeled (*see* **Note 1**).
2. Labeling medium: Dulbecco's Modified Eagle's Medium (DMEM) lacking methionine/cysteine, supplemented with 10 % dialyzed fetal bovine serum (FBS), 1× GlutaMax™, 110 mg/L Pyruvate, 1× Penicillin/Streptomycin (*see* **Note 2**). If the cells being used harbor a respiratory chain defect, media is also supplemented with 50 μg/mL uridine. The *de novo* synthesis of pyrimidines is linked to the electron transport chain by the enzyme dihydroorotate dehydrogenase, which uses ubiquinone as an electron acceptor. Therefore, cultured cells without a functional respiratory chain may become auxotrophic for uridine [17, 18]. Keep at 4 °C until use.
3. Regular DMEM: 10 % FBS and 50 μg/mL uridine (if required).
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4 (*see* **Note 3**).
5. 50 mg/mL chloramphenicol (CAP) in 100 % ethanol, store at −20 °C.
6. Cytosolic translation inhibitor:
   (a) *For pulse labeling*: 100 mg/mL emetine hydrochloride in 100 % ethanol, made fresh. Emetine hydrochloride is an irreversible inhibitor of the cytosolic ribosome.
   (b) *For pulse-chase labeling:* 100 mg/mL cycloheximide in 100 % ethanol or 20 mg/mL anisomycin in dimethylsulfoxide (DMSO), made fresh. Both cycloheximide and anisomycin are reversible inhibitors of cytosolic ribosomes and are critical for the post pulse-chase period.
7. [^{35}S]-labeled methionine/cysteine mix.

2.2 Mitochondrial Isolation from Cultured Cells

1. Solution A: 20 mM HEPES-KOH pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF; stock made to 100 mM in isopropanol and added fresh), and 2 mg/mL fatty acid-free bovine serum albumin (BSA).
2. Solution B: Solution A without the addition of BSA (*see* **Note 4**).
3. Sucrose storage buffer: 10 mM HEPES-KOH pH 7.6, 0.5 M sucrose.
4. Homogenizer and drill-fitted pestle.
5. Tris-SDS buffer: 50 mM Tris–HCl pH 8.0, 0.1 % (w/v) SDS.

2.3 In Organello Pulse Radiolabeling in Mitochondria Isolated from Tissues

1. Mitochondria isolation buffer for isolation from heart, muscle, and brain: 0.075 M sucrose, 0.225 M sorbitol, 1 mM EGTA, 0.1 % fatty acid-free BSA, 10 mM Tris–HCl, pH 7.4 supplemented with protease inhibitors (e.g. SIGMAFAST™ Protease Inhibitor).
2. Mitochondria isolation buffer for isolation from liver: 0.32 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4 supplemented with protease inhibitors (e.g. SIGMAFAST™ Protease Inhibitor).
3. Labeling Buffer A: 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 10 mM Tris–HCl, pH 7.4, 10 mM K_2HPO_4, 1 mg/mL BSA supplemented with protease inhibitors (e.g. SIGMAFAST™ Protease Inhibitor).
4. Labeling buffer B: Labeling Buffer A supplemented with 10 mM glutamate, 2.5 mM malate, 1 mM ADP, 1:100 amino acid mix, 1:100 cysteine, 1:50 tyrosine, 100 μg/mL cycloheximide.
5. Amino acid mix (6 mg/mL each in water): Ala, Arg, Asp, Asn, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, store at −20 °C.
6. Tyrosine (3 mg/mL in water), store at −20 °C.
7. Cysteine (6 mg/mL in water), store at −20 °C.
8. 2 M glutamate in water, store at −20 °C.
9. 1 M malate in water, store at −20 °C.
10. 0.5 M ADP in water, store at −20 °C.
11. 100 mg/mL cycloheximide, in water.
12. Homogenizer and drill-fitted pestle.
13. Scissors and forceps for tissue extraction.
14. [^{35}S]-labeled methionine/cysteine mix.

2.4 SDS-PAGE

For analysis of the radiolabeled proteins, we cast homemade Tricine SDS-PAGE gels using a SE600 Ruby gel electrophoresis system (GE Healthcare). The Tricine buffer system is used for the high resolution separation of proteins below 20–30 kDa [19], which includes many of the mtDNA-encoded subunits (Table 1). Alternatively, commercially supplied gels may be used in place of homemade gels.

1. SDS-PAGE loading dye: 50 mM Tris–HCl pH 6.8, 100 mM DTT, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue.
2. Acrylamide stock: 49.5 % (w/v) acrylamide/3 % (w/v) bisacrylamide, filtered.

Table 1
Relative molecular weight of mtDNA-encoded subunits from *Homo sapiens*

Subunit	Molecular weight (kDa)
Complex I	
ND1	35.7
ND2	37.0
ND3	13.1
ND4	51.6
ND4L	10.7
ND5	67.0
ND6	18.6
Complex III	
Cyt *b*	42.7
Complex IV	
COI	57.0
COII	25.6
COIII	29.9
Complex V	
ATP6	24.8
ATP8	8.0

3. Acrylamide gel solutions of 4, 10, and 16.5 % (w/v): Acrylamide diluted as required in 1 M Tris–HCl pH 8.45, 0.1 % SDS (*see* **Note 5**).
4. Cathode buffer: 100 mM Tris–HCl pH 8.25, 100 mM Tricine, 0.1 % SDS.
5. Anode buffer: 0.2 M Tris–HCl pH 8.9.

2.5 Blue Native (BN)-PAGE

The use of BN-PAGE was first described by Schägger and von Jagow [20] for the extraction of membrane complexes. Other options include different buffering systems including imidazole in place of bis–Tris [21]. We cast our blue-native gels in-house, however gels can also be purchased commercially for this application.

1. Solubilization buffer: 20 mM bis–Tris pH 7.4, 50 mM NaCl, 10 % (v/v) glycerol, plus an appropriate detergent.
2. Digitonin stock: 5 % (w/v) High purity Digitonin (Calbiochem), in water, dissolve completely by boiling briefly. Store at 4 °C until required.
3. Triton X-100 Stock: 10 % (v/v) Triton X-100.

4. *n*-Dodecyl-β-d-maltoside.
5. Recirculating refrigerated water bath to maintain temperature at 4 °C.
6. 10× BN loading dye: 5 % (w/v) Coomassie Blue G, 500 mM ε-amino *n*-caproic acid, 100 mM bis–Tris pH 7.0.
7. BN-PAGE gel solutions: Acrylamide stock (*see* Subheading 2.4) diluted to 4 % (w/v) and 13 % (w/v) in 50 mM bis–Tris pH 7.0, 66 mM ε-amino *n*-caproic acid (*see* **Note 5**).
8. Blue cathode buffer: 15 mM bis–Tris pH 7.0, 50 mM tricine, 0.02 % (w/v) Coomassie Blue G.
9. Clear cathode buffer: 15 mM bis–Tris pH 7.0, 50 mM tricine.
10. Anode buffer: 50 mM bis–Tris pH 7.

2.6 Post-electrophoresis Processing of Gels

1. Polyvinylidene fluoride membrane (PVDF).
2. PVDF Coomassie staining solution: 50 % (v/v) methanol, 10 % (v/v) acetic acid, 0.2 % (w/v) Coomassie Blue R.
3. PVDF destaining solution: 50 % (v/v) methanol, 10 % (v/v) acetic acid.
4. Phosphor imaging screen.
5. Phosphor image detector system.

3 Methods

3.1 Pulse and Pulse-Chase Labeling of mtDNA-Encoded Subunits in Cell Culture

This chapter will outline both the pulse and pulse-chase methods of mtDNA subunit labeling, highlighting the common steps and when these procedures differ. Figure 1 shows the radiolabeled mtDNA-encoded products following pulse-chase analysis as observed by both BN-PAGE (1A) and SDS-PAGE (1B).

1. *For pulse analysis*: The day before the experiment, cells are seeded into a number of 6-well (9.6 cm^2) plates (e.g. 6 different cell lines per plate and one plate per desired time point. Time points usually selected include 0 min (no [^{35}S] added), 30 min, 60 min, and 120 min at ~80–90 % confluency. Because generally whole cell lysates are used, sufficient material can be generated from a single well of a 6-well plate.

   For pulse-chase analysis: Early on the day of the experiment, cells are seeded onto either 94 mm or 145 mm culture plates, with one plate per time point desired (generally 3 time points suffice—0 h (directly after pulse labeling), 3, and 24 h post labeling).

   Allow cells to adhere during the day then supplement media with 50 μg/mL chloramphenicol for 15–18 h before the label-

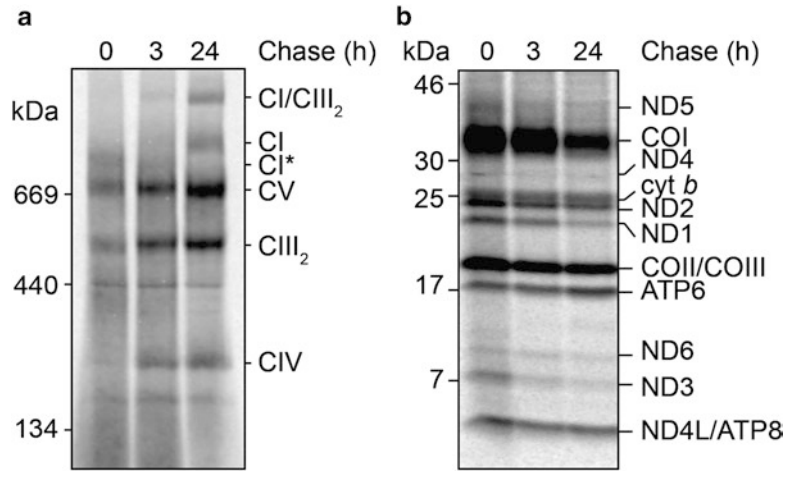

Fig. 1 Radiolabeling of mtDNA translation products from HEK293T cells. Following a 2-h pulse with [^{35}S]-Met/Cys to label mtDNA-encoded proteins, cells were chased for 0, 3, and 24 h to follow the assembly/stability of the radiolabeled subunits over time. (**a**) Isolated mitochondria were solubilized in 1 % Triton X-100 followed by BN-PAGE analysis. Complexes $CIII_2$, CIV, and CV accumulate over time. A complex observed at ~830 kDa (CI*) is gradually lost and coincides with the accumulation of mature complex I (CI) and the $CI/CIII_2$ supercomplex. (**b**) Isolated mitochondria were subjected to SDS-PAGE analysis. Signals for the radiolabeled proteins were detected for most of the 13 polypeptides encoded by mtDNA; subunits ND1-6 and ND4L of complex I, cyt *b* of complex III, COI-III of complex IV, and ATP6 and ATP8 of complex V

ing experiment is to commence. This will inhibit mtDNA translation and allow a pool of nuclear-encoded subunits to accumulate in the matrix before pulse labeling to stabilize the newly synthesized subunits [16, 22].

2. Rinse cells with warm phosphate-buffered saline (PBS) and then add half the regular amount of Met/Cys-free DMEM to the cells (*see* **Note 6**). Incubate at 37 °C and under 5 % CO_2 for 15 min.
3. Prepare labeling medium during this time. Again, the amount of media required depends on the scale of the experiment. For each sample, use half the regular amount of medium used for regular cell culture. Ensure the cytosolic translation inhibitor has been added. For *pulse analysis*, emetine hydrochloride is added to 100 μg/mL while for *pulse-chase analysis* cycloheximide or anisomycin is added to 10–100 μg/mL (*see* **Note 7**). Add the required amount and incubate at 37 °C and under 5 % CO_2 for 30 min.
4. Following this incubation, the [^{35}S]-Methionine/cysteine mix is added to the labeling media. The amount added is generally 12 μCi/mL (256 Ci/mmol/mL) and corresponds to 1 μL of

labeling mix per milliliter of labeling media (*see* **Note 8**). Cells are incubated at 37 °C and under 5 % CO_2 for the desired time.

5. *For pulse analysis*: Following the desired time, add cold (non-radioactive) methionine to a final concentration of 10 μM from a 10 mM stock and incubate at 37 °C for 10 min (*see* **Note 9**). The media is carefully removed and disposed of appropriately and the cells are rinsed once with pre-warmed PBS. The cells are then harvested by mechanical scraping, incubating briefly with trypsin, or PBS containing 1–5 mM EDTA for adherent cells. Suspension cells can be centrifuged at 800 × *g* after the desired time point. Store the cells on ice until all samples have been harvested.

   For pulse-chase analysis: Following the pulse labeling (generally carried out for 2 h), add cold (non-radioactive) methionine to a final concentration of 10 μM from a 10 mM methionine stock (*see* **Note 9**). Incubate at 37 °C and under 5 % CO_2 for 15 min. Following this, carefully remove media and rinse with ~3–10 mL PBS depending on the size of your plate. Replace with regular DMEM/FCS and commence the chase. Harvest a "0 h" time point as described above. Continue the chase for the desired time—usually a further 3 and 24 h before harvesting cells (s*ee* **Note 10**). Samples can be centrifuged and stored at −80 °C until use.

3.2 Mitochondrial Isolation from Cultured Cells

A simple method to isolate mitochondria is mechanical disruption of the cell membrane by homogenization, followed by differential centrifugation. To maintain protein integrity and minimize the activity of proteases, all steps are performed at 4 °C or on ice. The process to isolate crude mitochondria is as follows:

1. The harvested cell pellets from a 9 or 15 cm plate are resuspended in 3 or 5 mL of Solution A respectively. Cells are incubated on ice for 15–20 min allowing cell swelling to occur.
2. Following this, samples are homogenized using a drill-fitted Teflon pestle and a 5 mL glass homogenizer with approximately 15–20 strokes (*see* **Note 11**).
3. Non-lysed cells and nuclei are removed by centrifugation at 800 × *g* for 15 min at 4 °C.
4. The supernatant is then subjected to centrifugation at 10,000 × *g* for 20 min at 4 °C with the resulting pellet enriched for mitochondria.
5. The pellet is then washed by resuspending in 3 mL of Solution B followed by centrifugation (10,000 × *g*, 30 min at 4 °C).
6. The final pellet is then resuspended in 200–500 μL of Solution B or Sucrose storage buffer (for freezing) before determination of protein concentration.

7. The concentration of protein in isolated mitochondria can be determined by measuring the absorbance at 280 and 310 nm of 1 µL of the mitochondrial suspension in 600 µL of Tris-SDS buffer using a quartz cuvette. The mitochondrial protein concentration is then given by Clarke [23].

$$\text{protein concentration}\left(\text{mg}/\text{mL}\right) = \frac{\left(\text{A}_{280\text{nm}} - \text{A}_{310\text{nm}}\right)}{1.05} \times 600$$

Alternately, the protein concentration for whole cell lysate and mitochondria can be determined using commercially available kits.

3.3 Mitochondrial Isolation from Mouse Tissue

In contrast to the above-described method, the order of labeling and mitochondria isolation is reversed for the *in organello* analysis of protein synthesis. For this procedure, it is important that the isolated mitochondria are fully functional [24]. Therefore, the purification procedure is quick and gentle. It is important to keep samples at 4 °C or on ice to maintain functionality of mitochondria and to use the freshly prepared mitochondria on the same day without freezing or storing for the labeling (*see* **Note 12**).

1. Anesthetize mice and remove organs without previous perfusion (for muscle: both legs with upper and lower muscle, *see* **Note 13**).
2. Place the organs in a pre-chilled beaker, rinse briefly with cold PBS to remove debris and residual body fluid and add 6 mL of the appropriate mitochondrial isolation buffer. In case of skeletal muscle, add 6 mL per leg (total 12 mL).
3. Mince tissues while in the buffer using scissors. This step will make the following homogenization faster and easier, especially for muscular tissue.
4. Homogenize solution with the following setting: Liver/brain: 6 strokes at 600 rpm; heart/muscle: ten strokes at 600 rpm.
5. Transfer homogenates into a centrifuge tube and remove unbroken cells and debris by centrifuging at 1000 × *g*, 5 min, 4 °C. The mitochondria are in the supernatant.
6. Transfer the supernatant into 3 × 2 mL microfuge tubes and pellet mitochondria in a table top by centrifuging at 10,000 × *g*, 2 min, 4 °C.
7. Discard the supernatant and wash the mitochondria by resuspending each pellet with 500 µL isolation buffer. Pool and transfer in one 1.5 mL microfuge tube. Re-pellet mitochondria by centrifuging at 10,000 × *g*, 2 min, 4 °C.
8. The final pellet is then resuspended in 1 mL Labeling Buffer A and protein concentration is determined as described above.
9. The mitochondrial suspension is stored on ice until the labeling experiments starts.

3.4 In Organello Pulse Labeling of mtDNA-Encoded Subunits

This section describes the pulse labeling of mtDNA-encoded subunits in mitochondria freshly isolated from mouse tissues and is based on the methods described previously [24]. The profiles observed for mtDNA-encoded subunits of various tissues following *in organello* labeling of isolated mitochondria are shown in Fig. 2.

1. The volume equivalent to 1 mg protein freshly isolated mitochondria (*see* Subheading 3.3) is centrifuged at 10,000×*g*, 2 min, 4 °C and the pellet is resuspended in 500 μL Labeling Buffer B at 37 °C for 5 min while gently shaking.
2. Following this incubation, the [^{35}S]-Methionine labeling mix is added to the mitochondrial suspension (~75 μCi; ~7.5 μL) and the mixture is incubated for 1 h at 37 °C shaking at ~600 rpm (*see* **Note 8**).
3. The mitochondria are pelleted by centrifugation at 10,000×*g* for 2 min and resuspended in Labeling Buffer B to wash the mitochondria and stop the labeling reaction.
4. After labeling, the mitochondria are pelleted as above and resuspended in 100 μL 2× SDS-PAGE buffer. For denaturing, this suspension is incubated at RT for 30 min (do not boil).

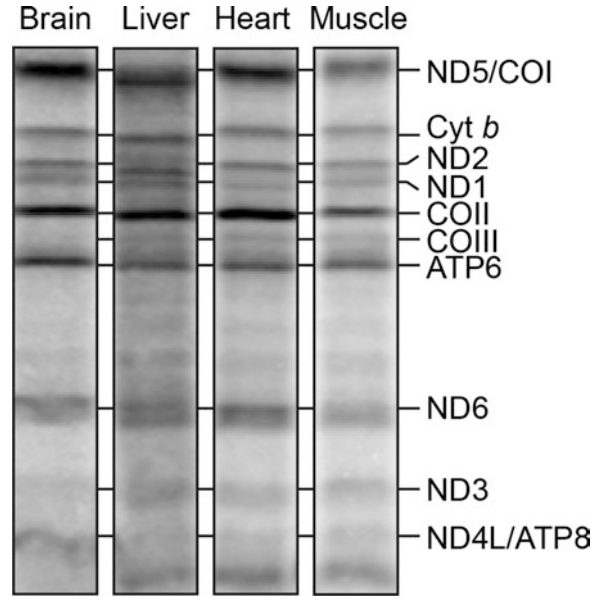

Fig. 2 Radiolabeling of mtDNA translation products in mitochondria isolated from brain, liver, skeletal muscle, and heart. Mitochondria are freshly isolated in a gentle procedure to maintain full functionality. Translation products are labeled by a 1 h pulse with [^{35}S]-Met in buffer containing the essential amino acids. The labeled mitochondria are denatured and subjected to SDS-PAGE analysis. Signals for the radiolabeled proteins were detected for most of the 13 polypeptides encoded by mtDNA; subunits ND1-3, -5, -6 and ND4L of complex I, cyt *b* of complex III, COI-III of complex IV and ATP6 and ATP8 of complex V

3.5 Analysis of Proteins Using SDS-PAGE

1. Once the protein concentration has been determined, the appropriate amount of sample is collected and pelleted.
2. For mitochondrial samples, this is generally 25–50 μg of protein, while for whole cell sample it is 70–100 μg.
3. For *in organello* labeling, typically 10–20 μL of the finally resuspended mitochondria is used (100–200 μg protein).
4. The pellets are resuspended in SDS-PAGE loading dye. Samples are not boiled but heated to 65 °C as the high heat may cause these highly hydrophobic proteins to aggregate and may lead to the loss of the signal for the radiolabeled protein [22].
5. The samples obtained from the *in organello* labeling are denatured by incubation at room temperature for 30 min.
6. To analyze mtDNA translation products, we use a Tris-tricine buffering system as described by Schägger and von Jagow [19]. A continuous 10–16 % acrylamide separating gel is prepared using a gradient gel mixer with 18 cm × 16 cm plates that fit into the SE600 Ruby electrophoresis system (GE Healthcare, UK), although other systems can be employed.
7. The stacking gel is prepared from the 4 % (w/v) acrylamide solution.
8. The samples are subjected to electrophoresis at 100 V/25 mA for 13–16 h at room temperature (*see* **Note 14**).

3.6 Analysis of Protein Complexes Using Blue Native (BN)-PAGE

1. Active protein complexes can be separated under native conditions using blue native (BN)-PAGE [20]. This technique utilizes mild conditions (including mild detergents such as Digitonin or Triton X-100, neutral pH, and cold temperatures) to extract and maintain the integrity of membrane protein complexes. We use the method described by Schägger and von Jagow [20] with some modifications.
2. To prepare samples for BN-PAGE analysis, cell pellets (100 μg total protein) or isolated mitochondria (30–70 μg) are solubilized in solubilization buffer containing an appropriate detergent to a final concentration of 1 μg protein/μL for 30 min on ice.
3. The choice of detergent should reflect the purpose of the desired experiment as different detergents will solubilize protein complexes to different extents. We commonly use 1 % (w/v) Digitonin which acts as a very mild detergent and is capable of maintaining the interactions between the respiratory complexes I, III and IV.

4. Alternately, the use of 1 % (v/v) Triton X-100 or *n*-dodecyl-β-d-maltoside (DDM) at concentrations 0.2–1 % (w/v) is able to separate the respiratory supercomplexes into individual holocomplexes or smaller supercomplexes for analysis [16].
5. Following solubilization, the sample is cleared of any insoluble material by centrifugation at 18,000 × *g* for 5 min at 4 °C. The cleared lysate is transferred to a fresh tube, taking care not to disturb the pellet (*see* **Note 15**).
6. To this, 5 μL of 10× BN-PAGE loading dye is added and briefly centrifuged at 4 °C.
7. For molecular weight markers, 20 μg each of thyroglobulin (669 kDa), ferritin (440 kDa), and bovine serum albumin (BSA; 134 and 67 kDa) is also applied to the gel.
8. For the analysis of respiratory chain complexes, we generally use 4–13 % (w/v) continuous gradient gels prepared from solutions of 4 % (w/v) and 13 % (w/v) acrylamide gel solutions using 18 cm × 16 cm plates that fit into a Ruby SE600 gel system (GE Healthcare, UK).
9. For electrophoresis, all buffers are chilled to 4 °C before use and maintained at this temperature using a refrigerated recirculating water bath set to 4 °C. This allows a low stable temperature to occur during the electrophoresis process.
10. Initially a "blue cathode buffer" is used until the samples have entered approximately one-third of the way into the gel. At this point the buffer is exchanged for the "clear cathode buffer" which does not contain Coomassie Blue G.
11. Electrophoresis is carried out at 100 V/10 mA for 13–14 h at 4 °C.
12. Once completed, gels are further processed as described below.

3.7 Analysis of Protein Complexes Using 2D BN/SDS PAGE

1. In order to investigate the subunit composition of multicomponent complexes, 2D BN/SDS PAGE can be employed [10, 12, 16].
2. To perform this analysis, BN-PAGE is carried out as described above.
3. Following this, the lane of interest is excised from the gel and placed between two glass plates and placed on an angle with the higher percentage end of the acrylamide slightly lower to allow more even entry of proteins from the BN-PAGE into the second dimension.
4. The SDS-PAGE separating gel (10–16 % gradient) is then poured as described previously below the BN-PAGE gel strip.

5. Once the separating gel has set, a 4 % (w/v) acrylamide sacker is poured around the gel strip, ensuring that air bubbles do not form at the interface of the gel strip and the stacking gel.
6. Electrophoresis is performed at 100 V/25 mA for 13–16 h at room temperature.

3.8 Processing of BN-, SDS-, and 2D BN/SDS-PAGE Gels

1. Following electrophoresis, the proteins can be fixed and the gel is then dried or the proteins can be transferred to a polyvinylidene fluoride (PVDF) membrane using the semi-dry western transfer method (nitrocellulose cannot be used for BN-PAGE) and following this, the membrane is allowed to dry completely.
2. Following transfer, proteins are stained briefly in Coomassie solution, followed by the removal of excess Coomassie using a destaining solution.
3. The dried gel or membrane is then exposed to a phosphor imaging screen for the desired amount of time (*see* **Note 16**).

4 Notes

1. Depending on the analysis required, using 145 mm culture dishes will provide sufficient material to conduct BN-PAGE, SDS-PAGE, and 2D BN/SDS-PAGE using isolated crude mitochondria.
2. Check formulation of DMEM, in particular DMEM lacking Methionine and Cysteine. Before adding components ensure they are not already present in the medium.
3. Phosphate buffered saline (PBS) can be made in-house, or it can also be purchased ready to use in liquid or tablet form from various suppliers.
4. Solution B can be made and stored at −20 °C until required. To make Solution A, add the required amount of BSA and PMSF fresh before use.
5. Gel solutions can be prepared beforehand and stored at 4 °C. These solutions can then be used to prepare gels on the day of the experiment.
6. During shorter incubations, using half the amount of medium is sufficient to cover cells and reduce the amount of reagents consumed.
7. It is recommended that the cytosolic ribosome inhibitor is titrated against the cell line of interest as some lines may be more sensitive than others to the treatment at a given concentration.

8. At this time all media and cell waste is regarded as a radioactive hazard and should be handled with care and disposed of appropriately.
9. This step is important to allow nascent polypeptides to complete translation. Without this step, there may be a large amount of background due to incorporation of the radioactive amino acids into proteins that may not have been fully translated after the pulse period.
10. Because the chase period can last up to 24 h, ensure to use media specific for the cell line being used.
11. If a drill-fitted pestle is not available, this step can be performed by hand using 30 strokes.
12. Work with animals including mouse breeding, anesthetizing and tissue preparation requires ethical permission from the local authorities.
13. The *in organello* labeling protocol works best for mitochondria freshly prepared from young mice (age: 3–6 months).
14. This gel system can be used with 15-well or 20-well combs. Preparing gels with 15 wells is recommended as this will increase resolution between samples as they will be better separated.
15. If the pellet is mistakenly applied to the gel, this will result in smearing of the sample. If in doubt, transfer to a fresh tube and repeat the centrifugation step.
16. For SDS-PAGE analysis, a strong signal will be evident after a few hours. For BN-PAGE and 2D-PAGE analysis, a number of days may be required for a stronger signal to be observed.

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Chapter 10

Analysis of Mitochondrial DNA Copy Number and Its Regulation Through DNA Methylation of *POLGA*

Xin Sun, William Lee, Vijesh Vaghjiani, and Justin C. St. John

Abstract

Replication of mitochondrial DNA (mtDNA) is important for ensuring that cells have sufficient mtDNA copy number to meet their specific requirements for the generation of cellular energy through oxidative phosphorylation. A number of transcription and replication factors are required for this process, with a key factor being the nuclear-encoded mtDNA-specific DNA polymerase γ. DNA polymerase γ has a catalytic subunit (POLGA), whose gene has been shown to be DNA methylated at exon 2. This methylation is considered to be one of the key mechanisms that regulate mtDNA copy number. These findings have made it of great importance to establish optimal methods for investigating the effects of DNA methylation on mtDNA replication. Here, we provide methods to determine the extent of DNA methylation at exon 2 of *POLGA* as well as other gene targets of interest. We also show how mtDNA copy number is assessed and, from these two outputs, define the efficiency of mtDNA replication by calculating the mtDNA-replicative efficiency index.

Key words mtDNA copy number, MeDIP, DNA methylation, POLGA

1 Introduction

Replication of mitochondrial DNA (mtDNA) is key to developmental outcome. To this extent, it has been demonstrated that mtDNA copy number is strictly regulated during the process of development, whereby copy number decreases significantly from the numbers present in the fertilized oocyte as it progresses through pre-implantation development [1]. This continues until the blastocyst stage, the final stage of preimplantation development, when the trophectoderm and the inner cell mass are established. These two cell populations then give rise to the placenta and the embryo proper, respectively [2]. The inner cell mass is also the source of embryonic stem cells, which have the potential to differentiate into all cell types of the body [3]. In the blastocyst, mtDNA replication is initiated in the trophectoderm. However, the cells of the inner

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cell mass do not replicate mtDNA. This remains the case until later stages of development when naïve embryonic cells commit to specific lineages [4]. Consequently, embryonic stem cells are excellent models to study mtDNA replication.

Regulation of mtDNA copy number is primarily under the control of the nuclear genome [5, 6]. Indeed, the factors that regulate mitochondrial transcription and replication are encoded by the nuclear genome [7]. Recently, it has been shown that one of these key factors, the mtDNA-specific polymerase, DNA polymerase γ (POLG), undergoes DNA methylation within the second exon of the gene encoding its catalytic subunit (*POLGA*) [8, 9]. This finding has been shown both in mouse [8] and human [10] embryonic and differentiating stem cells, and in tissues. Indeed, continuous replication of mtDNA is indicative of the ability of the naïve cell to differentiate and to generate ATP through oxidative phosphorylation [10], which takes place within the electron transfer chain. This is also most likely to be the case across a large number of species. DNA methylation at exon 2 has also been shown to be a regulator of mtDNA copy number in tumor cells [10].

DNA methylation can be determined through either bisulfite sequencing or immunoprecipitation of DNA using an antibody capture approach, known as *m e*thylated *D*NA *I*mmuno*p*recipitation (MeDIP) [8, 10]. The MeDIP approach enables *de novo* DNA methylation and demethylation to be assessed on the same sample using two antibodies. The first antibody captures 5-methylcytosine (5mC), which is indicative of *de novo* DNA methylation. The second antibody, 5-hydroxy methylcytosine (5hmC), enables *de novo* demethylated DNA to be captured. This approach has advantages over bisulfite sequencing, which does not distinguish between *de novo* DNA methylation and demethylation [10]. We have used antibodies to 5mC and 5hmC to assess the levels of DNA methylation at exon 2 of *POLGA* and shown that the ratio of the differences between the two populations of captured mtDNA can generate a mitochondrial replication efficiency index, when expressed in combination with mtDNA copy number [10]. Furthermore, it is worth mentioning that there is emerging evidence showing that mtDNA could be DNA methylated [11]. Even though this appears controversial, it is interesting to consider this point when investigating the regulation of the mitochondrial genome.

The assays we show here demonstrate how to capture *de novo* methylated DNA at exon 2 of *POLGA* and also from regions within the mitochondrial genome. We further show how to quantitatively assess the amount of methylated and non-methylated DNA as well as mtDNA copy number. From this, we demonstrate how to determine the mtDNA-replicative efficiency index so that a variety of cells from different stages of development and disease states can be accurately compared to determine whether mtDNA

copy number and the regulation of mtDNA replication through DNA methylation at exon 2 at *POLGA* are attributable to the disease being investigated. As it becomes evident which other mtDNA replication factors undergo DNA methylation, these assays can be easily applied to the affected gene regions.

2 Materials

2.1 Immunoprecipitation of Methylated DNA (MeDIP)

1. DNA sonication.
   (a) Covaris Adaptive Focused Acoustics (AFA™) S220 system.
   (b) Covaris milliTUBE.
2. DNA electrophoresis.
   (a) Agarose in 1× Tris-EDTA (TE) buffer (pH 8.0, 10 mM Tris, 1 mM EDTA).
   (b) Sybr safe DNA gel stain.
3. 10× IP buffer: 100 mM sodium phosphate pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100.
4. Immunoprecipitation.
   (a) Anti-5mC antibody.
   (b) Anti-5hmC antibody.
   (c) Dynabeads® Protein G.
5. Suspension mixer wheel.
6. PBS/BSA buffer: 0.1 % bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS).
7. Magnetic rack.
8. 10× Proteinase K digestion buffer: 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1.0 % Sodium dodecyl sulfate (SDS).
9. Proteinase K (20 mg/mL).
10. Thermo-shaker.
11. PCR purification kit.
12. Autoclaved Milli-Q water.
13. 1.7 mL sterile microcentrifuge tubes.

2.2 Quantifying MeDIP Products Using Real-Time PCR

1. SensiMix Hi-Rox real-time PCR mastermix.
2. Gene-specific primers.
3. Real-Time PCR instrument (*see* **Note 1**).
4. 0.1 mL sterile strip tubes and caps.

2.3 Determining mtDNA Copy Number

1. PCR reagents:
   (a) 10× NH_4 reaction buffer.
   (b) 50 mM $MgCl_2$ solution.
   (c) 50 mM dNTP mix dilution.
   (d) *Taq* polymerase.
2. Gene specific primers.
3. 0.2 mL sterile polypropylene PCR tubes.
4. Gel extraction kit.
5. SensiMix Hi-Rox real-time PCR mastermix.
6. Real-Time PCR machine.
7. 0.1 mL sterile strip tubes and caps.

3 Methods

3.1 MeDIP

1. Pre-cool and degas the Covaris Adaptive Focused Acoustics (AFATM) S220 system (*see* **Note 2**).
2. In a Covaris milliTUBE, aliquot 5–10 μg of genomic DNA and adjust to 100 μL with autoclaved Milli-Q water. Add 100 μL of 2× TE buffer. Sonicate the DNA sample in the Covaris Adaptive Focused Acoustics (AFA™) S220 system for 195 s to generate fragments sized between 200 and 1000 bp. The sonication setting is 5–10 °C for the temperature range, 105.0 for peak power, 5.0 for duty factor and 200 for cycles/burst.
3. To confirm efficient sonication, check the DNA by electrophoresis on a 1.5 % agarose gel. The DNA should appear as a smear between 200 and 1000 bp. If a smear is not present, repeat the sonication step.
4. In a new tube, add 3 μg of sonicated DNA and adjust to a final volume of 450 μL with autoclaved Milli-Q water.
5. Denature DNA samples by incubating at 95 °C for 10 min, then immediately place on ice for 5 min.
6. Add 50 μL of 10× IP buffer and mix well by pipetting.
7. Add 3 μg of anti-5mC or anti-5hmC antibody into each sample (1 μg per 1 μg DNA).
8. Incubate samples at 4 °C for 2 h by rotation.
9. Vortex Dynabeads® Protein G bottle thoroughly and immediately aliquot 30 μL per sample (10 μL per 1 μg DNA) into a new tube.
10. Pre-wash the beads with 1 mL PBS/BSA buffer for 5 min at 4 °C by rotation. Place the tube in a magnetic rack and remove the surrounding fluid and repeat the wash two more times. Each time, place the tube in the magnetic rack to remove the fluid from the beads.

11. Resuspend the beads in 30 μL of 1× IP buffer.
12. Add beads into each sample and incubate at 4 °C for 16 h by rotation (*see* **Note 3**).
13. Place samples in a magnetic rack for 1 min. Remove all fluid.
14. Wash the beads with 1 mL of 1× IP buffer for 5 min at 4 °C by rotation. Repeat the wash two more times. Place the samples in a magnetic rack. Remove all fluid.
15. Resuspend beads in 250 μL of proteinase K digestion buffer and add 10 μL of 20 mg/mL proteinase K.
16. Incubate at 50 °C for 3 h on a thermo-shaker.
17. On a magnetic rack, transfer the supernatant into a new tube.
18. Extract DNA using a PCR purification kit. Elute purified DNA in 50 μL of autoclaved Milli-Q water (*see* **Note 4**).

3.2 Quantifying MeDIP Products by Real-Time PCR

1. Real-time PCR is applied to quantify the levels of enrichment of 5mC and 5hmC in the gene of interest. This is achieved by comparing levels of 5mC and 5hmC in the gene of interest with a reference DNA sample (Input), which is prepared from the same DNA source but it only undergoes the DNA sonication and PCR purification steps in the MeDIP procedure (see above).
2. Run Input, 5mC and 5hmC samples for each DNA sample. Prepare sufficient sample to run reactions in triplicate. Prepare two reactions as non-template control (NTC).
3. Prepare reactions for real-time PCR in a total volume of 20 μL, containing: 10 μL of 2× SensiMix SYBR HI-ROX mastermix, 1 μL (5 μM) of each forward and reverse primer for the gene of interest (primer information for several mtDNA genes and exon 2 of *POLGA* shown in Table 1), 6 μL autoclaved Milli-Q water, 2 μL of purified MeDIP sample or H_2O as NTC.
4. Run each reaction on a real-time PCR instrument in triplicate. The reaction profile settings are: 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, followed by gene-specific annealing temperature (Table 1) for 15 s with an extension for 15 s at 72 °C (cycling A) and for 15 s at the product-specific melting temperature (cycling B; Table 1). Data are acquired from the Cycling B-FAM/Sybr channel (*see* **Note 5**).
5. Adjust the cycle threshold (CT) to obtain the CT values for each sample. The enrichment levels for 5mC or 5hmC are calculated using the following formula:

$$\text{Enrichment of 5mC} = 2^{(CT_{\text{Input}} - CT_{\text{5mC}})}$$

$$\text{Enrichment of 5hmC} = 2^{(CT_{\text{Input}} - CT_{\text{5hmC}})}$$

Table 1
qPCR primers for target genes

Human gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size	Annealing Tm	Cycling B. Tm
β-globin	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC	268	57	80
mtDNA	CGAAAGGACAAGAGAAATAAGG	CTGTAAAGTTTTAAGTTTTATGCG	152	53	78
POLGA exon 2	CAGACCTCCACGTCGAACAC	GACAACCTGGACCAGCACTT	209	59	83

6. To investigate if the mitochondrial genome is DNA methylated, regions of the mitochondrial genome can be quantified using MeDIP products (Table 2).

3.3 Determining mtDNA Copy Number by Real-Time PCR

1. Two standard curves need to be generated: one for nuclear DNA (*β-globin*) and one for mtDNA. All steps below should be applied to both nuclear DNA (*β-globin*) and mtDNA.
2. Adjust the concentration of total DNA templates to 100 ng/μL. For amplification of β-globin and mtDNA templates using conventional PCR, prepare the reaction mixture to a total volume of 50 μL containing: 5 μL of 10× NH_4 reaction buffer, 1.5 μL of 50 mM $MgCl_2$ solution, 0.5 μL 50 mM dNTP mix, 0.5 μL of *Taq* polymerase, 1 μL (25 μM) of each forward and reverse primer for β-globin or mtDNA (primer information shown in Table 1), 2 μL (200 ng) of total DNA and 38.5 μL autoclaved Milli-Q water.
3. Reaction profile settings are 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, primer-specific annealing temperature (Table 1) for 30 s and 72 °C for 30 s. The reaction is completed by an extension phase at 72 °C for 10 min.
4. To confirm DNA templates have been amplified, run PCR samples on a 2 % agarose gel.
5. Purify the targeted DNA by cutting the targeted band from the gel and extracting DNA with a gel extraction kit. Determine the amount of purified DNA template using a spectrophotometer.
6. Adjust the concentration of purified DNA templates to 1 ng/μL.
7. Prepare tenfold serial dilutions of the purified DNA from 10^{-1} to 10^{-8} ng/μL as the templates for the standards.
8. Adjust the concentration of the unknown DNA samples to 10 ng/μL.
9. Prepare quantitative PCR reactions to a total volume of 20 μL containing: 10 μL of 2× SensiMix SYBR HI-ROX mastermix, 1 μL (5 μM) of each forward and reverse primer for β-globin or mtDNA (primer information shown in Table 1), 2 μL of each standard template or unknown sample and 6 μL autoclaved Milli-Q water.
10. Reactions are performed in a real-time PCR machine. Reaction profile settings are: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, gene-specific annealing temperature (Table 1) for 15 s and extension for 15 s at 72 °C (cycling A) and for 15 s at the product specific temperature (cycling B; Table 1). Extension and data acquisition are performed on Cycling B-FAM/Sybr channel.

Table 2
Primers for mitochondrial genes

Human gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size	Annealing Tm
MT-ND1	TCTCACCATCGCTCTTCTAC	GGTTGGTCTCTGCTAGTGTG	350	57
MT-CYB	ATGACCCCAATACGCAAAACT	GGGAGGACATAGCCTATGAA	401	54
MT-CO2	CTGCTTCCTAGTCCTGTATG	GTCGGTGTACTCGTAGGTTC	235	56
MT-ND6	CCGCACCAATAGGATCCTCCCGA	GCATGGGGGTCAGGGGTTGAG	187	63
MT-ATP6	CAGTGATTATAGGCTTTCGCTC	GTGTTGTCGTGCAGGTAGAG	343	57

11. Melt data are acquired from 72 to 98 °C with a 60 s interval before the first step followed by 4 s for each subsequent step of 1 °C increase. The melt curve determines the specificity of the primers, where only one curve should be observed.
12. A standard curve is produced and indicates the efficiency of quantitative PCR. The efficiency should range between 0.95 and 1.
13. Concentrations of the mtDNA and nuclear DNA-specific products are calculated based on the standard curve.
14. mtDNA Copy number per cell is calculated using the formula shown below (*see* **Note 6**):

    mtDNA copy number per cell $= 2 \times N_{\text{mtDNA}} / N_{\beta\text{-globin}}$.

    As chromosomal DNA is diploid in cells, mtDNA copy number is doubled.

    N_{mtDNA} and $N_{\beta\text{-globin}}$ are determined by the formula:

$$N = ([\text{ng} / \mu\text{L}] \times 6.023 \times 10^{14}) / (\text{product size in bp} \times 660)$$

where N is the number of molecules per reaction, [ng/μL] is the concentration determined by the standard curve computationally, 10^{14} is the conversion of 1 mol to 1 nmol using Avogadro's constant, which states 1 mol $= 6.023 \times 10^{23}$, 660 is the mean molecular weight of a nucleic acid base pairing in Daltons (Da).

15. Replication efficiency index = mtDNA copy number/(enrichment level of 5mC/enrichment of 5hmC) (*see* **Note 7**).

4 Notes

1. Our reaction and analyses are conducted on a 72-well Rotorgene-3000 Real-Time PCR machine with Rotor-Gene 6 software. Different instruments can be used according to the prescribed operational manual.
2. For MeDIP, it normally takes 2 h for the sonication system to cool down and degas.
3. For MeDIP, it is important to plan the timeline well in advance because the procedure includes a series of incubation steps, especially a 16 h incubation step. We normally start this at 5 p.m. and terminate it at 9 a.m. the next day.
4. The MeDIP products are single-stranded DNA. For any downstream experiments requiring double-stranded DNA, second-strand synthesis should be performed.
5. Collection of data at cycling B ensures that background noise, such as primer-dimers, are excluded from the analysis.

Table 3
Example of real-time PCR dataset for mtDNA standards and samples

Name	Type	Average Ct	Given conc. (ng/μL)	Calc conc. (ng/μL)
STD1	Standard	6.19	0.1	
STD2	Standard	8.51	0.01	
STD3	Standard	11.73	0.001	
STD4	Standard	14.96	0.0001	
STD5	Standard	18.81	0.00001	
STD6	Standard	22.51	0.000001	
STD7	Standard	25.47	0.0000001	
H_2O	NTC		0	
Sample	Unknown	10.25		0.00742

6. An example of how to calculate mtDNA copy number:

   From the real-time PCR, the data collected from the Cycling B-FAM/Sybr channel (Table 3) are used to generate a standard curve, i.e., an output graph (Fig. 1). As shown in the output graph, the efficiency and R values met the requirements to qualify as standards. In the dataset, the NTC sample was not detected. Based on the standard curve, the concentration of the sample was given by computational analysis. As shown in Table 3, the mtDNA concentration of the sample is 0.00742 ng/μL. In the same way, the concentration for *β-globin* of the sample can also be determined.

   As an example, if the concentration of the *β-globin* gene is 0.000007 ng/μL, then:

$$N_{\text{mtDNA}} = (0.00742 \times 6.023 \times 10^{14}) / (152 \times 660) = 44560413.8$$

$$N_{\beta-\text{globin}} = (0.000007 \times 6.023 \times 10^{14}) / (268 \times 660) = 23835.9$$

$$\text{mtDNA copies per cell} = 2 \times N_{\text{mtDNA}} / N_{\beta-\text{globin}} = 3738.9$$

7. An example of how to calculate the replication efficiency index:

   MeDIP experiments will have determined the CT values for 5mC and 5hmC at, for example, 26.4 cycles and 27.2 cycles, respectively and for the Input at 25.3 cycles. Therefore:

$$\text{Enrichment of 5mC} = 2^{\left(\text{CT}_{\text{Input}} - \text{CT}_{\text{5mC}}\right)} = 2^{(25.3-26.4)} = 0.467$$

$$\text{Enrichment of 5hmC} = 2^{\left(\text{CT}_{\text{Input}} - \text{CT}_{\text{5hmC}}\right)} = 2^{(25.3-27.2)} = 0.268$$

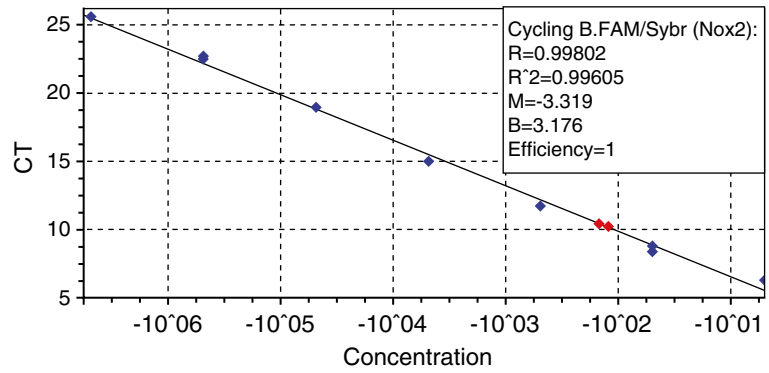

Fig. 1 Example of a standard curve generated for mtDNA copy number from real-time PCR

Also taking the mtDNA copy number determined above, as an example, therefore,

the mtDNA replication efficiency index for a specific cell or sample

=mtDNA copy number/(enrichment level of 5mC/enrichment of 5hmC)

=3738.9/(0.467/0.268)=2146

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Part IV

Modification of Mitochondrial DNA

Chapter 11

Engineered mtZFNs for Manipulation of Human Mitochondrial DNA Heteroplasmy

Payam A. Gammage, Lindsey Van Haute, and Michal Minczuk

Abstract

Enrichment of desired mitochondrial DNA (mtDNA) haplotypes, in both experimental systems and the clinic, is an end sought by many. Through use of a designer nuclease platform optimized for delivery to mitochondria—the mitochondrially targeted zinc finger-nuclease (mtZFN)—it is possible to discriminate between mtDNA haplotypes with specificity to the order of a single nucleotide substitution. Site-specific cleavage of DNA produces a shift in the heteroplasmic ratio in favor of the untargeted haplotype. Here, we describe protocols for assembly of paired, conventional tail–tail mtZFN constructs and experimental approaches to assess mtZFN activity in mammalian cell cultures.

Key words Genetic engineering, Zinc finger, Zinc finger nuclease, Mitochondrial disease, Heteroplasmy, Gene therapy

1 Introduction

Human mitochondrial DNA (mtDNA) is a 16.5 kb, circular, multi-copy genome encoding several structural components of the respiratory chain, ATP synthase, and RNA components necessary for mitochondrial gene expression. This mtDNA is contained within the mitochondrial matrix of all nucleated mammalian cells, associated with the inner mitochondrial membrane. Typically, there are between ~100–10,000 copies of mtDNA per cell, dependent on cell type, and accumulation of mutations within these molecules can occur at a rate approximately an order of magnitude greater than that of genomic DNA [1]. Consequently, sequence variation between mtDNA molecules within a cell occurs frequently, a state known as mtDNA heteroplasmy. Such variations, and other errors of mtDNA maintenance and metabolism, can produce either non-pathogenic polymorphisms or disease-causative point mutations and rearrangements of mtDNA. Mitochondrial disease originating from heteroplasmic mutation of

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the mitochondrial genome depends on the ratio of mutated:non-mutated mtDNA present in a cell. The heteroplasmic threshold required for disease presentation is variable between mutations, typically requiring ~60–90 % mutant mtDNA load. This threshold is dependent on mutation, tissue distribution and likely other, as yet unknown factors [2]. A significant majority of mtDNA-mediated mitochondrial disease is heteroplasmic in nature, with only a handful of less-severe mutations known to present in a homoplasmic fashion, where all of the mtDNA is mutated [3].

The heteroplasmic nature of mtDNA in the disease state allows an unusual gene therapy approach, entailing the selective targeting and degradation of mutant mtDNA molecules based on DNA sequence discrimination. Reducing mutant mtDNA copy number leads to replication of the spared wild-type molecules, as a result of the cell recovering to the initial, gross mtDNA copy number. The principle of mtDNA haplotype-specific targeting, degradation, and heteroplasmy shifting has been demonstrated in several systems over the past decade (Fig. 1a) [4, 5], and recently also through the use of mitochondrially targeted zinc finger-nucleases (mtZFN) [6].

Zinc finger-nucleases (ZFN) are chimeric DNA cleaving enzymes comprising a zinc finger protein (ZFP)-based DNA recognition domain [7] conjugated to a dimeric DNA cleavage domain at the c-terminus, derived from the type IIS restriction enzyme *Fok*I [8, 9]. By targeting pairs of ZFN in tail–tail orientation to adjacent DNA sequences, ZFN have been demonstrated to produce sequence-specific DNA double-strand breaks that can facilitate genetic manipulations across a range of cellular or animal model systems, and have led to the development of credible therapies, such as for HIV [10]. To manipulate heteroplasmy of mtDNA bearing a point mutation, a pair of mtZFN monomers are designed; a mutation-specific monomer (Fig. 1b, red), which binds a DNA sequence including the mutation, ensuring that this monomer binds only to mutated mtDNA molecules, and a mutation non-specific monomer (Fig. 1b, blue), which binds an adjacent DNA sequence contained within both wild-type and mutated mtDNA. Cleavage of mtDNA requires dimerization of the c-terminal *Fok*I domains, which, using this targeting strategy, will only occur on the mutant molecule. This technology can also be adapted to manipulate heteroplasmic deletions of mtDNA; by targeting mtZFN to positions partially overlapping a deletion breakpoint, and up or downstream sequence, it is possible to produce a pair of mtZFN that will only be adjacent, and dimerize, when bound to deleted mtDNA, but not to wild-type (Fig. 1c).

Our laboratory has developed, and is constantly refining mtZFNs to target mammalian mtDNA *in situ*. After the initial description of an efficient method to deliver ZFPs to mitochondria, we provided proof-of-principle that ZFPs can access and bind sites on human mtDNA with single nucleotide specificity [11]. Our ear-

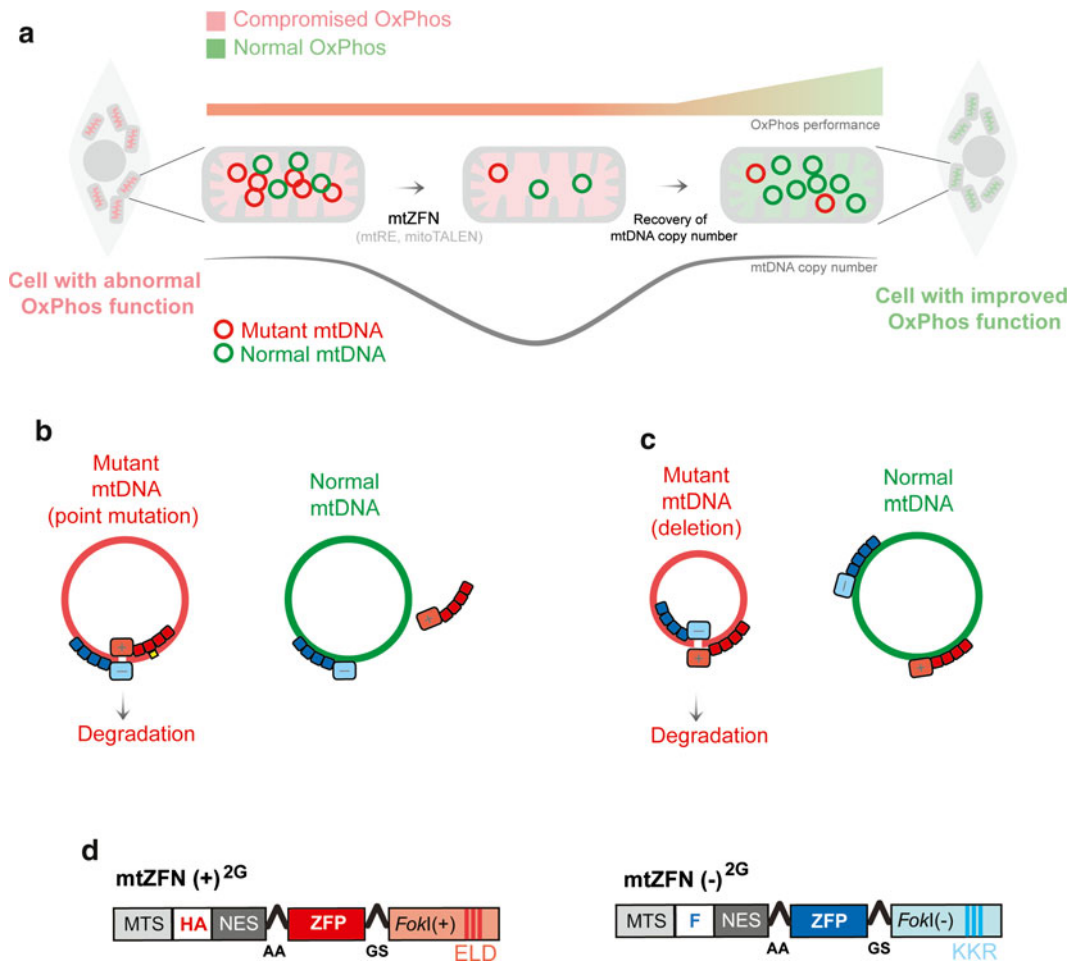

Fig. 1 Mitochondrially targeted ZFNs for manipulation of mtDNA heteroplasmy. (**a**) Approaches to treatment of mitochondrial DNA disease using engineered nucleases. In heteroplasmic cells with compromised OxPhos, mtZFN recognizes the mutant haplotype (*red*), with much greater specificity than normal mtDNA (*green*). Double-strand breaks lead to a preferential and rapid degradation of mutant mtDNA accompanied by a reduction in mtDNA copy number. In order to recover mtDNA copy-number, the residual, mostly wild-type, mtDNA replicates. This results in cells being repopulated by normal copies of mtDNA and subsequent improvements in OxPhos function. Similar approaches have been reported that use mitochondrially targeted restriction enzymes (mtRE, [4, 25]) or mitochondrial TALE-nucleases (mitoTALEN, [5]), instead of mtZFN. (**b**) Strategy for targeting mtDNA point mutations by mtZFN. The mutation-specific mtZFN monomer (*red* construct) recognizes the mutation site and dimerizes with the companion mtZFN (*blue* construct) that binds mtDNA on the opposite strand. Dimerization leads to DNA cleavage, followed by degradation of the mutant haplotype. mtZFNs do not dimerize on wild-type mtDNA. (**c**) Strategy for targeting mtDNA deletions by mtZFNs. mtZFN monomers dimerize at the deletion break-point leading to dsDNA breaks and subsequent degradation of mtDNA. Wild-type mtDNA is not cleaved as mtZFN monomers bind several kilobases apart, preventing dimerization. (**d**) Schematic of the obligatory heterodimeric mtZFN^{2G} monomers. Mitochondrial targeting is facilitated by a 49 amino acid *MTS* from subunit $F_1\beta$ of human mitochondrial ATP synthase. *NES* nuclear export signal. *F* FLAG tag and *HA* hemagglutinin tag. *ZFP* zinc finger peptide. "+" and "−"—ELD and KKR modified *Fok*I domain, respectively [14]. *AA* two-alanine linker. *GS* glycine-serine linker [9]

lier designs for dimeric mtZFNs were unreliable in selectively targeting and degrading mitochondrial genomes in living cells, despite high activity and specificity *in vitro* [12, 13]. To overcome this, a single-chain mtZFN, which carries two cleavage domains linked to the same protein, was designed [12]. The single-chain mtZFN allowed for specific targeting of mtDNA point mutations, however, high levels of off-target cleavage were observed. More recently we have designed and reported an improved, second generation of mtZFNs (mtZFN^{2G}), designed to eliminate human mtDNA molecules with pathogenic point mutations and large-scale deletions. A key improvement in this architecture was incorporation of modified *Fok*I domains that cleave DNA only when paired as a heterodimer (Fig. 1d mtZFN(+)^{2G} and mtZFN(−)^{2G}). These *Fok*I variants exhibit efficiency comparable to the wild-type architecture, but with a >40-fold reduction in homodimer activity [14–16]. Degradation of mutant mtDNA by mtZFN^{2G}, combined with concomitant replication of wild-type mtDNA molecules, was shown to produce large shifts in mtDNA heteroplasmy [6].

In this chapter we describe methods for assembly and testing of tail–tail oriented mtZFN of our latest architecture, capable of targeting and selectively degrading virtually any desired mtDNA sequence, producing large, specific shifts in mtDNA heteroplasmy. It is assumed that, prior to commencing these protocols, a cytoplasmic hybrid (cybrid) cell line bearing a mutation of interest, and a library of ZFPs specific to this mutation, have been acquired. Suitable published protocols are available for ZFP design [17–20] and cybrid generation [21]. As an example case throughout this chapter, we will be using cells bearing the m.8993T>G point mutation and ZFPs specific to this particular mutation, as used in previously published work from our laboratory [6, 12].

2 Materials

2.1 Molecular Cloning and Plasmid DNA Preparation

1. pcDNA3.1(−) vector.
2. pTracer CMV/bsd vector.
3. pcDNA3.1(−)_mCherry vector.
4. KOD DNA polymerase.
5. Nuclease-free water.
6. Restriction endonucleases and buffers, various.
7. T4 DNA Ligase.
8. DNA oligonucleotides (*see* Table 1).
9. Commercial Gel Extraction kit.
10. Commercial PCR Purification kit.

Table 1
DNA oligonucleotides for ZFP cloning

Primer	Primer sequence
*EcoR*I_ZFP_FWD	5′ TACGAATTCATGGCTGAGAGG 3′
*BamH*I_ZFP_REV	5′ ATTGGATCCCCGCAGGTGTATC 3′

11. 5× DNA Loading Dye.
12. Agarose.
13. SYBR Safe DNA gel stain.
14. 1 kb plus DNA ladder.
15. 1× TBE electrophoresis buffer: 900 mM Tris–HCl, 900 mM boric acid, 20 mM EDTA, pH 8.0.
16. Subcloning efficiency DH5α competent cells.
17. TYE bacterial medium.
18. Ampicillin.
19. Commercial Miniprep kit.
20. Commercial Plasmid Midiprep kit.

2.2 Cybrid Cell Transfection and Fluorescence-Activated Cell Sorting (FACS)

1. Tissue culture plastics: 75 cm^2 flasks, 6-well plates, 15 mL conical tubes.
2. 5 mL polystyrene round-bottom tube.
3. CellTrics cell-suspension filter.
4. 1× DMEM, supplemented with 4.5 g/L d-glucose, Glutamax and 100 mg/L sodium pyruvate.
5. 1× PBS, without $CaCl_2$ or $MgCl_2$.
6. 10× Trypsin-EDTA solution.
7. Fetal calf serum.
8. Opti-MEM, reduced serum media.
9. Lipofectamine 2000.
10. BD FACSAria III FACS sorter.

2.3 Protein Extraction and Immunoblotting

1. Western lysis buffer: 50 mM Tris-acetate pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) Triton X-100, 1× proteinase inhibitor cocktail.
2. BCA Protein Assay kit.
3. Spectrophotometer.
4. 4× NuPAGE LDS sample buffer.
5. DTT.

Table 2
Antibodies required for western blotting

Epitope	Dilution
Primary antibodies	
HA	1:500
FLAG	1:2,000
β-actin	1:100,000
Secondary antibodies	
Goat anti-mouse IgG HRP	1:2,000
Goat anti-rat IgG HRP	1:1,000

6. NuPAGE Novex 4–12 % bis–tris gels.
7. XCell SureLock Mini-Cell.
8. 20× MES SDS running buffer.
9. Trans-blot semi-dry transfer cell.
10. 1× Semi-dry transfer buffer: 25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol.
11. Coomassie stain.
12. Non-fat milk powder.
13. Antibodies, various (*see* Table 2).
14. 1× Western incubation buffer: 1× PBS, 5 % (w/v) non-fat milk powder.
15. 1× Western washing buffer: 1× PBS, 0.1 % (w/v) Triton X-100.
16. 1× Stripping buffer: 62.5 mM Tris base, 100 mM beta-mercaptoethanol, 2 % (v/v) SDS.
17. Nitrocellulose membranes.
18. ECL detection reagents.
19. 1-mm cassettes.
20. X-ray film.

2.4 mtDNA Heteroplasmy and Copy Number Measures

1. KOD DNA polymerase.
2. Nuclease-free water.
3. User designed synthetic DNA oligonucleotides and qPCR probes (*see* Table 3).
4. Radiolabeled dCTP [α-^{32}P]-3000 Ci/mmol^{-1}, 10 mCi/mL^{-1}, 250 μCi.

Table 3
DNA oligonucleotides and probes required for qPCR

Primer/probe	Sequence
B2M F	5′ TGCTGTCTCCATGTTTGATGTATCT 3′
B2M R	5′ TCTCTGCTCCCCACCTCTAAGT 3′
B2M Probe	6-Fam-TTGCTCCACAGGTAGCTCTAGGAGG-Tamra
mt3211 F	5′ CACCCAAGAACAGGGTTTGT 3′
mt3298 R	5′ TGGCCATGGGTATGTTGTTAA 3′
mt3242 Probe	6-Fam-TTACCGGGCTCTGCCATCT-Tamra
mt9827 F	5′ CGTCATTATTGGCTCAAC 3′
mt9974 R	5′ GATGGAGACATACAGAAATAG 3′
mt9852 Probe	6-Fam-ACTATCTGCTTCATCCGCCACTAA-Tamra

5. *Sma*I restriction endonuclease.
6. DE81 ion exchange paper.
7. Gel dryer.
8. PhosphorImager (Typhoon 9400, GE Healthcare) or X-ray film with developing system.
9. 2× TaqMan gene expression master mix.
10. MicroAmp Optical 96-well reaction plates.
11. MicroAmp Optical Adhesive Film.
12. 7900HT Fast Real-Time PCR system.

2.5 General Laboratory Equipment

1. 0.5 mL centrifuge tubes.
2. 1.5 mL centrifuge tubes.
3. Standard PCR thermal cycler.
4. DNA mini gel tank.
5. Power Pack.
6. Nanodrop 2000 Spectrophotometer (or similar).
7. Tube roller.
8. Tilt platform.
9. Benchtop centrifuge.
10. UV transilluminator.
11. Chemiluminesence Detection System.

2.6 Non-commercial Biological Materials

1. Human osteosarcoma line 143B, NARP m.8993T>G cybrid [22] (kindly donated by Prof. Eric Schon).

3 Methods

Carry out all procedures at room temperature unless otherwise stated. All solutions are prepared using ultrapure (deionized, purified) water and analytical grade reagents, and can be stored at room temperature unless otherwise indicated.

3.1 Assembly of mtZFN Monomers and Expression Vector Cloning

Using pcDNA3.1(−)_mtZFN(+)^{2G}/pcDNA3.1(−)_mtZFN(−)^{2G} architectures available from Addgene, the pre-generated library of paired ZFPs must be cloned into their respective +/− backbones. There is no beneficial effect of having the mutation-specific or mutation non-specific monomer in either mtZFN(+) or mtZFN(−) backbone, though throughout this protocol, mutation-specific ZFPs are cloned into the mtZFN(+) backbone and mutation non-specific ZFPs are cloned into the mtZFN(−) backbone.

1. PCR amplify the ZFP sequences in a 50 μL reaction using KOD DNA polymerase, according to the manufacturer's instructions, using the ZFP_Fwd and ZFP_Rev primers. This produces a PCR product containing the ZFP with 5′ *Eco*RI and 3′ *Bam*HI flanking restriction sites (*see* Table 1).
2. From this PCR reaction, take 5 μL and resolve on a 1.2 % agarose gel, run at 7 V/cm for 1 h. If the PCR achieves decent product amplification (*see* **Note 1**), purify the remainder of the PCR product using a commercial PCR purification kit, and precipitate DNA overnight, resuspending in 20–40 μL nuclease-free water, giving a final concentration of >100 ng/μL as determined by spectrophotometer.
3. Using 10 μL of the purified PCR product, and both mtZFN(+) and mtZFN(−) backbone constructs, prepare double restriction enzyme digest reactions with 10 U *Eco*RI and *Bam*HI, according to the manufacturer's instructions. Resolve these digested fragments on 1.2 % agarose gels run at 7 V/cm for 1 h. Visualize DNA using a UV light box and excise fragments from the gel using a scalpel blade. Using a commercial gel extraction kit according to the manufacturer's instructions, recover DNA from these gel slices and measure concentration of eluted DNAs by spectrophotometer.
4. Ligate chosen ZFPs into either mtZFN(+) or mtZFN(−) backbone using T4 DNA ligase, according to the manufacturer's instructions (*see* **Note 2**). Take 5 μL of the ligation reaction, incubate with DH5α competent cells and transform using the

heat shock method, plating cells on dishes of TYE media supplemented with 50 μg/mL ampicillin and incubating at 37 °C overnight. Select an appropriate number of resulting colonies and amplify in TYE broth supplemented with 50 μg/mL ampicillin at 37 °C overnight in an incubator with shaking platform. After overnight incubation, isolate plasmid DNA using a commercial spin miniprep kit according to the manufacturer's instructions. Repeat *EcoR*I–*BamH*I double digestion and agarose gel electrophoresis from **Step 3** using isolated plasmid DNA to confirm presence of ZFP within the pcDNA3.1(−)_mtZFN(+/−) backbone, then confirm by Sanger sequencing using CMV forward and BGH reverse priming sites located adjacently to the multiple cloning site (MCS) of pcDNA3.1(−).

5. Once pcDNA3.1(−)_mtZFN(+) and pcDNA3.1(−)_mtZFN(−) backbones have been confirmed as containing the appropriate ZFPs by sequencing, the entire transgene for each monomer must be recloned into vectors capable of co-expressing mtZFNs and one of two fluorescent reporter proteins simultaneously in mammalian cells (*see* **Note 3**). The vectors used in this capacity are pcDNA3.1(−)_mCherry (pcmCherry) and pTracer CMV/Bsd (pTracer), co-expressing mCherry and cycle-3GFP, respectively; mtZFN(+) is cloned into pcmCherry and mtZFN(−) is cloned into pTracer (Fig. 2a, b). The MCS of both expression vectors is flanked at the 5′ and 3′ ends by *Pme*I restriction sites, as is the MCS contained within the original pcDNA3.1(−)_mtZFN(+/−) architecture.
6. To reclone the mtZFN transgenes, digest 2 μg of expression vectors and mtZFN(+) and mtZFN(−) constructs with 10 U of *Pme*I, according to the manufacturer's recommendation, then resolve the digested fragments on a 0.8 % agarose gel run at 7 V/cm for 1 h and excise fragments using a scalpel blade. Repeat gel extraction, ligation, transformation, digestion screening, and sequencing confirmation steps as laid out in **Steps 4** and **5** (*see* **Note 4**). Once clones containing mtZFN(+/−) transgenes within correct expression vectors have been obtained, produce a larger, high concentration plasmid DNA stock for both mtZFN(+/−)-containing vectors and vectors not containing a transgene; the "empty" vectors are used as a control condition in further experiments. Such stocks can be obtained by using a commercial plasmid midi kit, as directed by the manufacturer.

3.2 Testing mtZFN in Cybrid Cells

The efficacy of mtZFN pairings, in terms of mtDNA heteroplasmy and copy number, can be tested by various experimental methods in mammalian cell cultures. Prior to this, an enriched population of cells expressing both monomers, or control vectors, must be obtained. In our experience, the most efficient and robust method

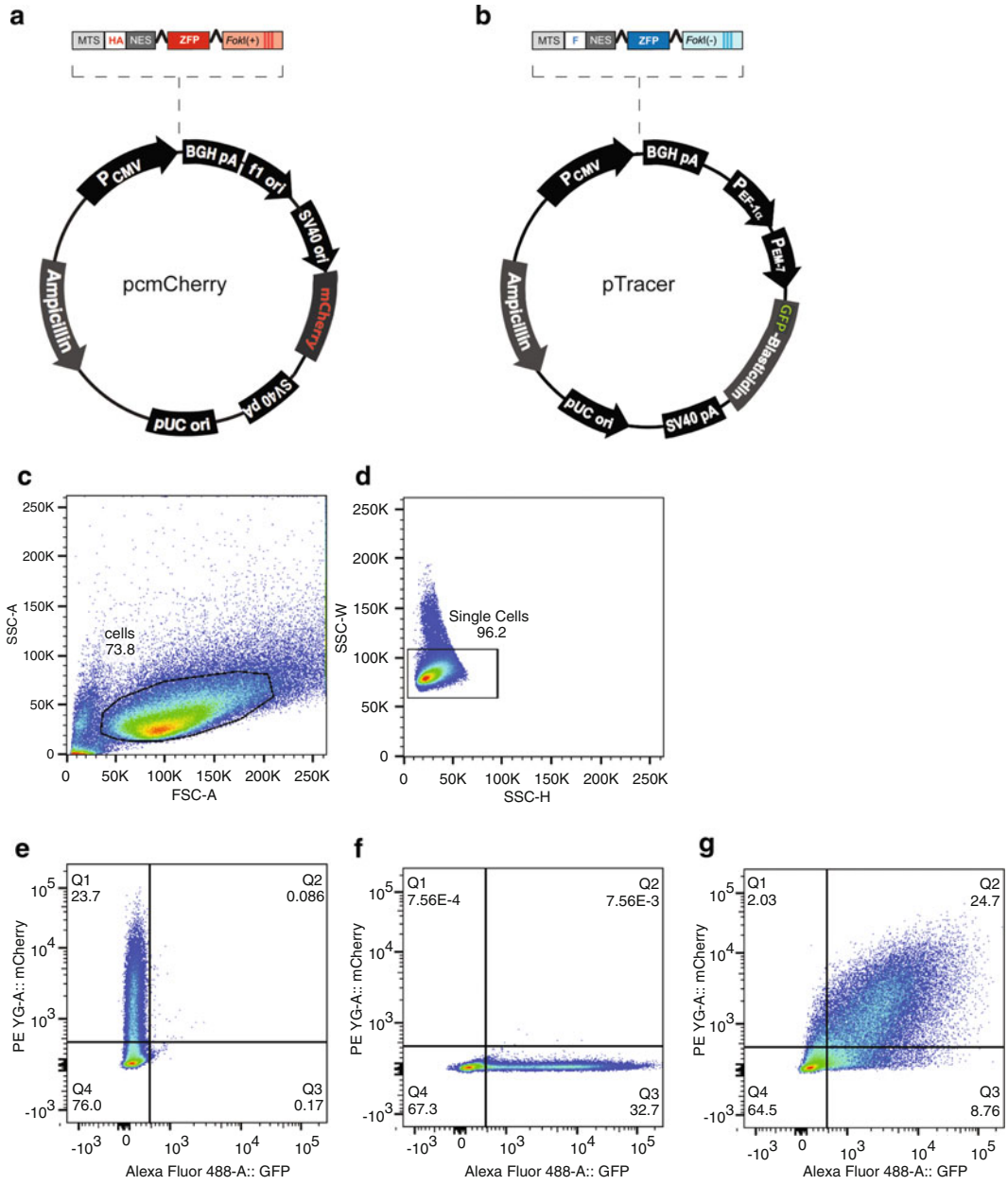

Fig. 2 Fluorescent reporter co-expression vectors and FACS gating strategy. (**a**, **b**) Schematic vector maps are shown. mtZFN^{2G} monomers were cloned into pcmCherry and pTracer vectors, which co-express the transgene and either mCherry or GFP fluorescent marker proteins. mtZFN(+) constructs were cloned into pcmCherry and mtZFN(–) constructs were cloned into pTracer. (**c**–**g**) Flow cytometry dot-plot graphical read-outs. Each point represents a cell or "event". (**c**, **d**) Dot plots depict forward scatter (FSC) and side scatter (SSC) profile of a typical sample for HOS 143B cells. FSC indicates cell diameter. SSC is a measure of light scattering from an orthogonal position to that provided by FSC. SSC provides information about the granularity of cells, which, in conjunction with information from FSC, allows for gating of monodisperse, live cells in the sample. A typical gating strategy is shown in black lines. (**e**) Dot plot of cells transfected with pcmCherry. The population of mCherry-positive cells is contained within section Q1 of the plot. (**f**) Dot plot of cells transfected with pTracer. The population of GFP-positive cells is contained within section Q3 of the plot. (**g**) Dot plot of cells transfected with both pcmCherry and pTracer. The population of cells expressing both mCherry and GFP is contained within section Q2. Cells from this quadrant are dually transfected, and are sorted for later analysis

to achieve this is a dual-transfection fluorescence-activated cell sorting (FACS) approach, followed by further confirmatory analyses of mtZFN expression and activity. The key advantage of this strategy is that transient, high-level expression of mtZFN is produced within cells in a reproducible manner, both between and within experiments. Throughout the protocols set forth below, cultured cells are maintained in standard conditions (37 °C, 5 % CO_2) using DMEM supplemented with 10 % FCS (v/v), 4.5 g/L d-glucose, Glutamax and 100 mg/L sodium pyruvate.

3.2.1 Transfection and FACS

1. On the day prior to transfection, seed cells at a density of 2×10^6 cells per 75 cm^2 flask and leave overnight to attach. On the day of transfection, aspirate the original media, pipette fresh media onto plates, and transfect cells with a total of 20 μg of plasmid DNA (10 μg pcmCherry_mtZFN(+), 10 μg pTracer_mtZFN(−), or 10 μg of either empty vector) using Lipofectamine 2000, according to the manufacturer's instructions. After 6 h, aspirate media containing transfection reagents and replace with standard culture medium. Experimental conditions required for accurate FACS gating and robust interpretations of mtZFN expression and efficacy are provided in Table 4 (*see* **Note 5**).
2. At 24 h post-transfection, wash cells with 1× PBS, trypsinize and then collect cells in 15 mL tubes by centrifugation at 300 × *g* for 5 min. Resuspend the cell pellet in 750 μL 1× PBS and transfer to 5 mL round-bottomed tube by passing through a CellTrics strainer (*see* **Note 6**). It is essential that all samples are kept at 4 °C from this point.

Table 4
Experimental conditions for FACS gating and interpretation

Condition	Plasmid(s) transfected	Purpose
Untransfected	None	FACS gating parameters for untransfected cells
+mCherry	pcmCherry	FACS gating parameters for mCherry-positive cells
+GFP	pTracer	FACS gating parameters for GFP-positive cells
Vectors	pcmCherry/pTracer	Experimental negative control
mtZFN(+)	mtZFN(+)/pTracer	Monomer specificity control
mtZFN(−)	pcmCherry/mtZFN(−)	Monomer specificity control
mtZFN pair	mtZFN(+)/mtZFN(−)	Experimental condition

3. Load the untransfected control cells into the FACS sorter. After selecting monodisperse, live cells, based on forward and side scatter profile (Fig. 2c, d), set fluorescence gating using the untransfected and single-stained GFP/mCherry control transfectants (Fig. 2e, f), then load and sort experimental samples, retrieving only dual positive cells (Fig. 2g). For the data shown, GFP signal was excited by a 488 nm laser and emission detected by photomultiplier tube (PMT) with 530/30 band-pass and 502 long-pass filters. mCherry signal was excited with 561 nm laser and emission detected by PMT 610/20 band-pass and 600 long-pass filters. Samples must be sorted in a sterile environment and collected in sterile tissue culture media in autoclaved 1.5 mL tubes, which are immediately placed on ice once obtained. Typically, ~5×10^5 cells sorted per sample is required to complete all analyses (*see* **Note 7**).

3.2.2 Culture of mtZFN-Enriched Cells

Samples acquired by FACS must be divided into three groups in a ratio of 2:2:1. These groups are: (i) Cells for continuous culture, allowing observations of mtZFN effects over time. (ii) Cells to provide a DNA sample at the 24 h post-transfection time point. (iii) Cells to provide a protein sample, allowing detection of mtZFN expression at the 24 h post-transfection time point.

1. With group (i) cells, intended for continuous culture, pipette each sample into 1 well of a 6-well plate, topping up media to ~2 mL, and leave overnight in a tissue culture incubator to recover. There is likely to be significant cell death in all samples, due to the FACS process, and it is necessary to aspirate media and replace with fresh media on the following day. These cells should now be maintained and passaged as required until 18 days and 28 days post-transfection, when they can be subjected to the biological material extraction and biochemical analytic steps laid out here in Subheading 3.2.2, or in Subheadings 3.2.3 and 3.2.4.
2. With group (ii) cells, intended for DNA analyses, pellet cells in 1.5 mL tube by centrifugation at 300 × *g* for 5 min. Aspirate media and extract total DNA using a commercial blood and tissue DNA extraction kit according to the manufacturer's recommendation. Elute DNA in a small volume of nuclease-free water, typically ~30–50 μL, and measure DNA concentration by spectrophotometer.
3. With group (iii) cells, intended for protein analyses, pellet cells in a 1.5 mL tube by centrifugation at 300 × *g* for 5 min. Aspirate media and resuspend pellet in 20 μL western lysis buffer in a 0.5 mL tube. Incubate on ice for 20 min, then add 7.5 μL NuPAGE LDS sample buffer and 1.5 μL 1 M DTT, mix thoroughly by vortex and boil the sample for 5 min. At this point the sample can either be stored indefinitely at −20 °C, or analyzed by western blotting, as detailed in Subheading 3.2.3.

3.2.3 Detection of mtZFN Expression

1. To analyze samples for mtZFN expression by western blotting, load the entire sample from group (iii), and molecular weight markers, onto a 10-well NuPAGE SDS 4–12 % bis-tris gel and run at 200 V in 1× MES buffer for ~45 min.
2. Once appropriately resolved, remove the gel from its casing, soak in 1× semi-dry transfer buffer and electrotransfer proteins to nitrocellulose membranes at 1 mA/cm^2 for 105 min in a semi-dry transfer cell. Following transfer, wash the membrane briefly in 1× PBS and then block in 1× PBS with 5 % non-fat milk (w/v) (PBS-M) for 1 h at room temperature on a roller. Incubate the gel in Coomassie stain for several hours, and allow to partially de-stain in water for several further hours; this allows quick, reliable visualization of protein loading and efficiency of transfer (*see* **Note 8**). Image this gel, and then discard.
3. Once the membrane has been blocked, remove blocking solution, apply primary antibody in 1× PBS-M and incubate overnight on a roller at 4 °C. On the following day remove the primary antibody solution and wash the membrane three times with 1× PBS with 0.1 % Triton X-100 (w/v) (PBS-T) for 10 min on a roller at room temperature, and then incubate with the secondary antibody in 1× PBS-M on a roller for 1 h at room temperature. Remove the secondary antibody solution and wash the membrane three times with 1× PBS-T for 10 min, then develop using ECL reagents and expose to X-ray film in a dark room. A list of all primary and secondary antibodies used for western blotting is given in Table 2 (*see* **Note 9**).

3.2.4 Assessing the Effects of mtZFN on mtDNA

Measurements of mtDNA heteroplasmy and copy number can be made by various means. Invariably, the appropriate method for analysis of mtDNA contents will change, based on the haplotype studied and available techniques. Measurements of heteroplasmy and copy number in our laboratory typically exploit restriction fragment length polymorphism (RFLP), quantitative PCR (qPCR), Southern blotting and pyrosequencing analyses, though numerous further methods are available, and may be preferred, depending on circumstance.

3.3 Measuring mtDNA Heteroplasmy

Changes in heteroplasmy of the m.8993T>G mutation are measured by amplification of a portion of mtDNA containing the mutation using a last cycle hot PCR, incorporating radiolabeled nucleotides in the final PCR cycle to negate heteroduplex formation. The radiolabeled PCR products are then purified and analyzed by RFLP, based on the unique *Sma*I restriction site created by the m.8993T>G mutation. For robust interpretation of this assay, digestion controls bearing 100 % wild-type and 100 % mutant

DNA must be included (*see* **Note 10**). In this protocol, we use 143B wild-type and N^{100} as controls, bearing exclusively wild-type or m.8993T > G mtDNA, respectively.

1. Using the DNA samples obtained from group (ii), prepare 50 μL PCR reactions for each sample, containing 100 ng of total cellular DNA extract as template. Prepare three extra reaction volumes of master mix: two for 143B wild-type and N^{100} digest control conditions, and one further to store on ice. Set the thermal cycler to repeat the PCR program for a total of 15 cycles. Once this is complete, transfer PCR tubes to ice and proceed to an area appropriate for use of radioactive materials (*see* **Note 11**).
2. Taking the additional reaction volume, add 2 μL dCTP [α-^{32}P] and mix well, then pipette 5 μL of this master mix into each sample tube, mixing thoroughly, and then place in a thermal cycler for one final PCR cycle. Once this is complete, purify PCR products using a commercial PCR purification kit according to the manufacturer's recommendation, with the exception of using two 500 μL wash steps, rather than a single 700 μL step (*see* **Note 12**) and elute PCR products in 50 μL nuclease-free water. At this stage, PCR products can be stored for up to 1 week at −20 °C, though this can be varied when making downstream adjustments for the radioactive decay half-life of ^{32}P.
3. Prepare 10 μL *Sma*I digest reactions for all samples, including digest controls 143B and N^{100}, according to the manufacturer's instructions. Allow reaction to proceed for 2 h at 25 °C, then resolve on a 1 % agarose gel run at 7 V/cm for 50 min, and dry gel onto DE81 paper using gel dryer. Expose the dried gel to a phosphor screen overnight and image using the PhosphorImager (Fig. 3).

3.4 Measuring mtDNA Copy Number

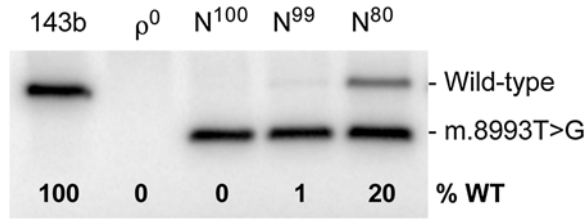

Fig. 3 Analysis of m.8993T > G mtDNA heteroplasmy in mammalian cell lines by last cycle hot RFLP. A representative result using DNA samples from cell lines bearing various mtDNA haplotypes: 143B (wild-type), ρ^0 (no mtDNA), N^{100} (100 % m.8993T > G), N^{99} (99 % m.8993T > G), and N^{80} (80 % m.8993T > G)

Quantification of gross mtDNA content in samples is achieved using qPCR, with the ratio of amplification from two mitochondrial amplicons compared individually with a nuclear control gene, *B2M*. In order to minimize any error introduced by unreliable DNA amplification or quantification by qPCR, the average of these two comparisons is taken as the measure of mtDNA copy number. Prior to measuring mtDNA copy number, the efficiency of the primers and probes should be tested by making standard dilution curves of known concentration. This test can be performed on an unrelated DNA sample, as long as the extraction method is the same as described previously. Standard dilution curves should be performed for each experiment when new primers or probes are used, or whenever appropriate (e.g. when not used for a significant period of time).

1. Dilutions are measured in triplicate in 25 μL reactions using the TaqMan gene expression master mix, as directed by the manufacturer, with 900 nM forward and reverse primers and 250 nM probe (*see* Table 3). Cycling conditions are: initial 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of: 95 °C for 15 s followed by 1 min annealing and extension at 60 °C.
2. To determine mtDNA copy number of the samples, 10 ng of total cellular DNA is used as template (*see* **Note 13**) and all samples are amplified in quadruplicate. Once amplification has been performed and data acquired, calculate relative quantities of PCR product using the $2^{-\Delta\Delta CT}$ method [23].

4 Notes

1. Owing to the highly repetitive modular sequence of ZFPs, some misamplification of PCR products is possible. To address this issue, add 5 % or 10 % DMSO (v/v) to your PCR reactions.
2. On ligations: (a) T4 DNA ligase buffer contains ATP, which is extremely sensitive to freeze–thawing cycles. To address this, aliquot buffer and avoid using previously thawed stocks. (b) T4 DNA ligase is sensitive to the chaotropic agent guanidium thiocyanate, contained in buffer QG from the Qiaquick gel extraction kit. To ameliorate the impact of this on ligation efficiency, ensure that the total volume of gel-extracted material present in ligation reactions does not exceed 20 % of the volume, i.e. 4 μL.
3. At this stage, prior to re-cloning into fluorescent reporter co-expressing vectors, mitochondrial localization of mtZFNs can be confirmed by confocal microscopy as described previously [13, 24]. This step is not strictly necessary, but may be useful

if issues with detection of mtZFN activity on mtDNA heteroplasmy are encountered, as inefficient delivery of the construct could be a plausible explanation.

4. As *Pme*I digestion produces blunt DNA ends, half of successful ligations will result in insertion of transgenes in the opposite, undesired orientation. Sequence several clones to ensure acquisition of a correctly oriented transgene.
5. Addition of propidium iodide to control cells (untransfected or single fluorescence) prior to sorting can allow for more specific identification of dead cells, and gives more accurate gating, though this is not strictly necessary. The addition of single fluorescence controls is critical to setting gates, allowing for maximum cell sorting efficiency. Without these controls, FACS operators must be far more conservative with gating, and this could result in an insufficient number of cells sorted.
6. When using the cell strainer, first pipette several mL of 1× PBS into an empty tube before passing your sample through. This prevents large volumes of your sample remaining within the mesh of the strainer.
7. The numbers of cells required for analyses are based on estimates that would allow for completion of all experiments from a single round of sorting. If only analysis of the effects of mtZFN after 24 h was required, for example, then the total number of sorted cells required can be decreased by ~2×10^5.
8. Use of cell number as a proxy for BCA acquired protein concentration values prior to western blotting can be helpful, especially when handling very small samples. The minimum number of cells that it is possible to generate data in this regard is ~2.5×10^4.
9. As it is likely that only one blot can be prepared from each FACS sort, the order of antibody probing is important, in order to avoid excessive stripping of the blot and loss of signal. The order used in our laboratory is: HA, FLAG, strip, B-actin. In the described conditions, HA signal is much weaker than that produced by FLAG (HA can require several minutes of exposure to generate a decent image, compared with a maximum of ~10 s for FLAG).
10. Digestion controls can either be DNA extracted directly from cell lines or, if cybrids bearing 100 % mutant mtDNA are not available, sub-cloned PCR products bearing the desired mtDNA haplotype, ligated into vectors and expanded in *E. coli*. Plasmids containing such sub-cloned portions of mtDNA can then be used as template for last-cycle hot PCR reactions and digested in control reactions.
11. The number of cycles required to reach the detection threshold for any one cell line is likely to vary considerably. For example, in our laboratory, the number of cycles required to

reliably detect mtDNA heteroplasmy by RFLP in m.8993T > G primary fibroblasts is ~20, but only ~15 in 143B cybrids. Run several PCRs of variable cycle number to ascertain the detection limit for the cell line of choice.

12. To avoid radioactive contamination of equipment, particularly centrifuges, it is highly recommended that collection tubes are discarded after each spin, and replaced with fresh tubes.
13. It is recommended that DNAs be diluted in nuclease-free water immediately prior to analysis.

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Chapter 12

Generation of Xenomitochondrial Embryonic Stem Cells for the Production of Live Xenomitochondrial Mice

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Abstract

The unique features of the mitochondrial genome, such as its high copy number and lack of defined mechanisms of recombination, have hampered efforts to manipulate its sequence to create specific mutations in mouse mtDNA. As such, the generation of *in vivo* mouse models of mtDNA disease has proved technically challenging. This chapter describes a unique approach to create mitochondrial oxidative phosphorylation (OXPHOS) defects in mouse ES cells by transferring mtDNA from different murid species into *Mus musculus domesticus* ES cells using cytoplasmic hybrid ("cybrid") fusion. The resulting "xenocybrid" ES cells carry OXPHOS defects of varying severity, and can be utilized to generate live mouse models of mtDNA disease.

Key words Mouse, Xenocybrid, Fusion, Fibroblasts, Embryonic stem cells

1 Introduction

Various methodologies for manipulating mtDNA have been explored to create live disease models. Some of the first experiments fused cytoplasts from NZB/BINJ mouse oocytes to single-cell C57BL/6 or BALB/c embryos [1–3], or enucleated eggs with membrane-bound karyoplasts containing a zygote nucleus and a portion of the oocyte cytoplasm [2, 4, 5], to generate heteroplasmic animals. An alternative approach used microinjection of *Mus spretus* somatic cell mitochondria directly into *Mus musculus domesticus* oocytes [6, 7] to generate heteroplasmic animals.

Cytoplast fusion was also used by the Hayashi group to create one of the first mtDNA disease models (mito-mouse) [8]. Synaptosome cybrids heteroplasmic for a 4,696 bp mtDNA deletion (originally captured from aged mouse brain) were enucleated and fused to pronucleus-stage embryos. The mtDNA deletion was transmitted through the female germline for three successive generations, with the percentage of deletion increasing with each generation.

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An alternative method for introducing different mtDNAs into live mice is ES cytoplasmic hybrid ("cybrid") fusion. Two independent groups used this approach to generate transmitochondrial mice carrying the mouse chloramphenicol-resistant CAP^R mutation [9]. CAP^R mutant cell lines were enucleated and the cytoplasts fused to ES cells, which were subsequently injected into blastocysts, and chimeric mice generated with detectable levels of the CAP^R mutation in various tissues [10, 11].

The Wallace group extended this approach by pre-treating the ES cells with the mitochondria-toxic dye rhodamine 6-G (R6G) to remove the endogenous mtDNA prior to cytoplast fusion, thereby greatly enriching the CAP^R mtDNA in the ES cell cybrids [11]. A female ES cell line that would produce fertile oocytes and transmit introduced mtDNA into the mouse germline was also identified. Using the R6G pre-treatment and the female CC9.3.1 ES cell line, chimeric mice were produced with high percentages of the CAP^R mutation that exhibited various ocular pathologies, while transmission of the CAP^R mutation through the mouse germline in a heteroplasmic or homoplasmic state resulted in progeny that died *in utero* or in the neonatal period, or live mice with progressive myopathy and dilated cardiomyopathy [12].

Following on from the "Mito-Mouse" mtDNA deletion model, the Hayashi group developed transmitochondrial mtDNA mutant mice that carry mutations in tRNA(Lys) [13] or *MT-ND5* [14, 15], both of which were originally identified in mouse tumor cells. The Wallace group have also developed other mtDNA mutant mice that harbor a *MT-ND6* mutation, resulting in a phenotype that mimics Leber Hereditary Optic Neuropathy (LHON) in humans [16]. Both groups have also generated mice that carry a *MT-CO1* mutation [17, 18] that was originally isolated from a L929 mouse cell line [19], while Weiss and colleagues utilized an mtDNA variant from the FVB mouse strain to create a conspecific C57Bl/6 mouse with an *MT-ATP8* point mutation that exhibits elevated mitochondrial ROS generation and pancreatic beta-cell dysfunction [20].

These models have utilized mtDNA mutations from aged mice, mouse strains or mouse cell lines; however, there is a lack of other mouse mtDNA mutations available in cell culture for the development of new live mtDNA mutant mice. To generate OXPHOS defects due to mtDNA variation, we have used an alternative approach by introducing mtDNA from different murid species into cells with a *Mus musculus domesticus* nuclear background. This mismatch of nuclear and mtDNA creates multiple OXPHOS defects that range in severity relative to the evolutionary distance of the mtDNA donors [21, 22]. While this approach cannot model point mutations that mimic human mtDNA diseases, it does enable exploration of whole animal consequences of increasing cellular stress from graded OXPHOS defects. The muridae family of

rodents has the largest number of species of any mammalian family, and so many xenocybrid constructs are possible.

Here, we describe a protocol for the transfer of mtDNA from a donor cell line (in this case, mouse L-cell fibroblast cybrids that were used to capture mtDNAs from different species) into female CC9.3.1 ES ES cells using a cybrid fusion approach. This protocol also describes how to deplete the ES cells of their endogenous mtDNA with Rhodamine-6G (R6G) before fusion, allowing for the generation of homoplasmic xenomitochondrial ES cells, and the subsequent handling and genotyping of the resulting ES cell xenocybrids. These ES xenocybrids can be used for *in vitro* differentiation experiments [23] or are suitable for blastocyst injection to generate live xenomitochondrial mice [24].

2 Materials

1. DMEM medium: DMEM (high glucose, glutamine, pyruvate) supplemented with 5 % fetal bovine serum (FBS), 1× Glutamax (Life Technologies), 1× penicillin/streptomycin and 50 μg/mL uridine.
2. Selection medium: DMEM supplemented with 5 % dialyzed FBS, 1× Glutamax, 1× penicillin/streptomycin and 50 μg/mL BrdU.
3. ES medium: DMEM (high glucose, glutamine, pyruvate) supplemented with 15 % ES cell grade FBS, 1× non-essential amino acids, 10^3 units/mL LIF, 1× β-mercaptoethanol, 1× Glutamax, 1× penicillin/streptomycin and 50 μg/mL uridine.
4. ES Freeze medium: ES medium supplemented with an additional 5 % ES cell grade FBS (for a total of 20 %) and 10 % DMSO.
5. 2,5-Bromodeoxyuridine (BrdU): 5 mg/mL stock in sterile H_2O.
6. β-Mercaptoethanol (1000× stock): Commercially available β-mercaptoethanol liquid is supplied as a 14.3 M stock. To 7.15 mL DMEM media, add 50 μL of 14.3 M stock (in fumehood). Filter sterilize through a 0.2 μm filter. Store wrapped in foil at 4 °C for up to 2 weeks.
7. Leukemia Inhibitory Factor (LIF): Can be obtained at 10^9 or 10^6 units/mL stocks. Working concentration in ES medium is 10^3 units/mL.
8. Hypoxanthine/Aminopterin/Thymidine (HAT): Supplied as a 50× stock.
9. Rhodamine-6G (R6G): 500 μg/mL stock in 2 % ethanol. Dissolve in absolute ethanol, dilute and filter sterilize through a 0.2 μm filter and store wrapped in foil at 4 °C.

10. Mitomycin c (20× stock): To 1 vial of mitomycin c powder (2 mg) add 5 mL of DMEM medium. Dissolve completely and transfer to a 10 mL tube. Make up to 10 mL with DMEM media and filter sterilize with a 0.2 μm filter. Store wrapped in foil at 4 °C for up to 4 weeks.
11. Cytochalasin B: 2 mg/mL stock in DMSO.
12. Polyethyleneglycol (PEG): We routinely use a 50 % w/v solution, cell culture fusion grade.
13. Percoll: Supplied as liquid.
14. Phosphate buffered Saline (PBS): Sterile 1× solution.
15. Trypsin/EDTA: 0.25 % Trypsin/0.25 % EDTA.
16. MtDNA donor fibroblasts (*see* **Note 1**).
17. Mouse L-cell ρ^0 fibroblasts (*see* **Note 2**).
18. Mouse ES cells (*see* **Note 3**).
19. Feeder cells such as mouse embryonic fibroblasts (MEFS) (*see* **Note 4**).
20. Sterile 50 mL polysulfone centrifuge tubes.
21. Sterile 6 mm × 8 mm glass cloning cylinders.
22. Sterile high vacuum grease.

3 Methods

3.1 Creation of Mouse L-Cell Xenocybrids: Fusion #1

Mouse xenocybrid fibroblasts are produced by enucleation of mtDNA donor cells (e.g. *Mus spretus* primary fibroblasts) and fusion of the resulting cytoplasts with mouse L-cell ρ^0 fibroblasts (Fig. 1).

1. In sterile 50 mL polysulfone tubes, prepare a solution containing 10 mL DMEM medium, 10 mL Percoll and 20 μg/mL cytochalasin B.
2. Grow mtDNA donor fibroblasts in DMEM medium and harvest approximately 5×10^6 cells. Resuspend cells in the medium/Percoll solution in the polysulfone tubes. Mix well, then spin at 19,000 rpm ($44{,}000 \times g_{av}$) for 70 min at 37 °C. Set temperature of centrifuge at 24 °C, which will result in an internal tube temperature of 37 °C.
3. Collect the cytoplast/karyoplast mixture from the medium/Percoll interface carefully using a 5 mL pipette. Harvest around 3–4 mL of mixture into a sterile 15 mL tube.
4. Dilute cytoplast/karyoplast mixture to 15 mL with DMEM medium, mix thoroughly to dilute the Percoll, then pellet cytoplasts/karyoplasts by centrifugation at $1{,}000 \times g$ for 10 min at room temperature. Remove medium carefully so that the cytoplast/karyoplast pellet is not disturbed.

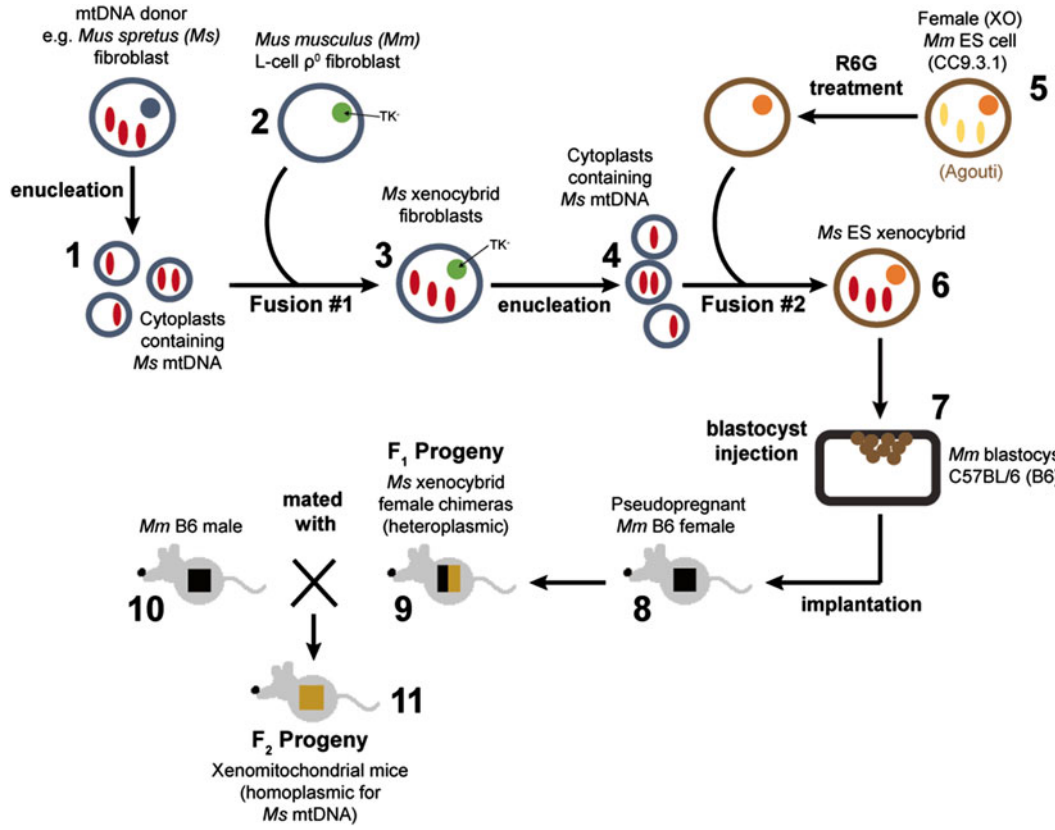

Fig. 1 Generation of live xenomitochondrial mice. This schematic is based on methods described for the generation of mouse xenocybrid fibroblasts [22] and live animals [24]. Cytoplasts containing the mtDNA of interest, for example *Mus spretus (Ms)* mtDNA, are generated by enucleation of donor primary fibroblasts [1]. These are fused (Fusion #1) to *Mus musculus (Mm)* mtDNA-less mouse L-cell ρ^0 fibroblasts [2] to create new *Ms* xenocybrid fibroblasts that contain *Mm* nDNA and *Ms* mtDNA [3]. To generate xenocybrid embryonic stem (ES) cells, the *Ms* xenocybrid fibroblasts [3] are enucleated to generate cytoplast donors containing *Ms* mtDNA [4]. These are then fused (Fusion #2) to female ES cells (CC9.3.1, derived from agouti mice) that have been pretreated with R6G to remove their endogenous mtDNA [5], creating new *Ms* ES cell xenocybrids [6]. The *Ms* ES cell xenocybrids are injected into *Mm* C57BL/6 (B6) blastocysts [7] which are then implanted into pseudopregnant *Mm* B6 females [8]. First-generation (F_1) females [9] that are heteroplasmic for *Ms* mtDNA are selected by coat color chimerism and bred with homoplasmic *Mm* B6 males [10]. Second-generation (F_2) mice with agouti coat coloring [11] will be homoplasmic for the donated *Ms* mtDNA, resulting in live xenomitochondrial mice

5. Grow the mouse L-cell ρ^0 fibroblasts in DMEM medium, harvest and count cell numbers. Approximately 2×10^6 ρ^0 fibroblasts are required for each fusion. Wash ρ^0 fibroblasts in 1× PBS, then resuspend in 20 mL of DMEM medium in a sterile 50 mL polysulfone tube. Use this medium to also resuspend the cytoplast/karyoplast pellet and transfer to the same polysulfone tube.
6. Spin the combined ρ^0 fibroblasts and cytoplasts/karyoplasts at $10{,}000 \times g$ for 10 min at room temperature.

7. Remove medium carefully to leave a dry pellet for fusion. Add 0.5 mL of PEG to the side of the polysulfone tube, then roll the PEG onto the ρ^0 fibroblast/cytoplast/karyoplast pellet and leave for 1 min. Roll PEG off the pellet and remove.
8. Resuspend the fusion pellet in DMEM medium and plate onto mitomycin c-treated feeder cells in 10 cm dishes at ~1×10^5, ~5×10^5, and ~1×10^6 ρ^0 fibroblasts per dish.
9. After 24 h, add selection medium, with medium replaced every 2–3 days for the next 10 days as clones appear (*see* **Note 5**).
10. Pick individual cybrid clones using a preferred method, for example cloning cylinders. Expand each clone and freeze for genotyping. These new xenocybrid fibroblasts can be used as the mtDNA donors for later ES cell fusion experiments (Subheading 3.4).

3.2 Preparing Feeder Cells for ES Culture

All ES cells must be grown on mouse feeder cells that have been pre-treated with mitomycin c to inhibit mitosis. These cells can be prepared as required or treated with mitomycin c in advance and frozen down for later use.

1. Grow feeder cells to confluency in 3 large T175 flasks.
2. Treat with 1× mitomycin c in DMEM medium for 3 h at 37 °C/5 % CO_2.
3. Wash cells in 1× PBS, then harvest with Trypsin (mitomycin c media waste must be collected and disposed of appropriately).
4. Cells can be plated for use the following day or frozen in vials at ~6×10^5 cells/vial (enough for 6 wells of a 6-well plate).

3.3 ES Cell R6G Treatment

1. Thaw out MEF feeders into a 10 cm dish the day before you wish to grow your ES cells.
2. The next day, thaw out CC9.3.1 ES cells in ES medium and grow for 2–3 days until ES cell clones are visible.
3. Begin treatment with 1 μg/mL R6G in 10 mL of ES medium for 72 h (*see* **Note 6**). Change treatment medium every 24 h.
4. After 72 h treatment, remove R6G treatment medium, wash cells gently in 1× PBS, then replace with 10 mL normal ES medium for 2 h before fusions.

3.4 Creation of Mouse ES Cell Xenocybrids: Fusion #2

Mouse ES xenocybrids are produced by enucleation of mtDNA donor cells (e.g. mouse L-cell xenocybrid carrying *Mus spretus* mtDNA) and fusion of the resulting cytoplasts with R6G-treated mouse ES cells (Fig. 1).

1. Grow mtDNA donor cells in a T175 flask. Approximately 10 times the number of donor cells to R6G-treated ES cells is required for the fusion experiment, i.e. ~6×10^7 donor cells for fusion with ~6×10^6 R6G-treated ES cells.

2. In sterile 50 mL polysulfone tubes, prepare a solution containing 10 mL DMEM medium, 10 mL Percoll and 20 μg/mL cytochalasin B.
3. Harvest donor cells and resuspend in the medium/Percoll solution in the polysulfone tubes. Mix well, then spin at 19,000 rpm ($44{,}000 \times g_{av}$) for 70 min at 37 °C. Set temperature of centrifuge at 24 °C, which will result in an internal tube temperature of 37 °C. This should be determined empirically for the refrigerated centrifuge used, to avoid an end run tube temperature greater than 37 °C which may lower cellular viability.
4. Collect the cytoplast/karyoplast mixture from the medium/Percoll interface carefully using a 5 mL pipette. Harvest around 3–4 mL of mixture into a sterile 15 mL tube.
5. Dilute cytoplast/karyoplast mixture to 15 mL with DMEM medium, mix thoroughly to dilute the Percoll, then pellet cytoplasts/karyoplasts by centrifugation at $1000 \times g$ for 10 min at room temperature. Remove medium carefully so that the cytoplast/karyoplast pellet is not disturbed.
6. Harvest the R6G-treated ES cells and count cell numbers. Approximately 6×10^6 ES cells are required for each fusion. Wash ES cells in 1× PBS, then resuspend in 20 mL of ES medium in a sterile 50 mL polysulfone tube. Use this medium to also resuspend the cytoplast/karyoplast pellet and transfer to the same polysulfone tube.
7. Spin the combined R6G-treated ES cells and cytoplasts/karyoplasts at $10{,}000 \times g$ for 10 min at room temperature.
8. Remove medium carefully to leave a dry pellet for fusion. Add 0.5 mL of PEG to the side of the tube, then roll the PEG onto the ES cell/cytoplast/karyoplast pellet and leave for 1 min. Roll PEG off the pellet and remove.
9. Resuspend the ES fusion pellet in ES medium and plate onto mitomycin c-treated feeder cells in 6-well plates at ~1×10^6, ~2×10^6, and ~3×10^6 ES cells/well.
10. After 24 h, add fresh ES medium, with medium replaced every 24 h for the first 2–3 days, then every 2–3 days for the next 10 days as clones appear. If the mitochondrial donor cells have a thymidine kinase negative (TK^-) nuclear background, then 1× HAT can be added to the ES medium to remove contaminating donor cells (*see* **Note 7**).

3.5 Picking Clones

Around 10–14 days after fusion, ES cybrid clones should be large enough to be picked from the fusion plate. We routinely use glass cloning cylinders and trypsin digestion (as described here), however mechanical dissociation or other suitable methods can also be used.

1. Prepare a 24-well tissue culture plate with mitomycin c-treated feeder cells the day before you plan to pick the ES cell colonies. Sterilize some cloning cylinders and high vacuum silicone grease by autoclaving. We routinely use 6 mm × 8 mm glass cloning cylinders to pick ES cell colonies.
2. Wash ES cells with 1× PBS, then remove. Dip the cloning cylinder into the vacuum grease using sterile forceps, then place around the colony of interest, being careful not to get grease on the cells.
3. Add 50 μL of trypsin into the cloning cylinder and incubate at 37 °C/5 % CO_2 for 3 min. Use a microscope to check that the ES cells have begun to dissociate from the plate surface.
4. Gently resuspend the cells in the trypsin and transfer onto mitomycin c-treated feeder cells in ES medium. Place one ES colony per well on the 24-well plate.

3.6 Harvesting ES Cell Clones for Freezing and Genotyping

1. Monitor growth of clones in 24-well plates. Clones will be ready to harvest in approximately 7–10 days.
2. When ready, wash with 500 μL 1× PBS, then add 200 μL trypsin and incubate at 37 °C/5 % CO_2 for 5 min.
3. Add 1 mL of ES medium and mix well with a 1 mL pipette tip to dissociate all ES cells.
4. Remove 1 mL of cells for freezing, leaving 200 μL to reseed well (these cells will be harvested later for genotyping).
5. Pellet 1 mL of ES cells by centrifugation at 300 × *g* for 3 min, remove medium and replace with 300 μL of ES Freeze medium and freeze using standard protocols. This will be passage number 3 (P3).
6. Add 1 mL of ES medium to the remaining 200 μL of trypsinized cells to reseed each well and continue growth of ES cell colonies.
7. After an additional 7 days, ES cells should be ready to harvest for genotyping.
8. Wash with 500 μL 1× PBS, then add 200 μL trypsin and incubate at 37 °C/5 % CO_2 for 5 min.
9. Add 1 mL of ES medium and mix well with a 1 mL pipette tip to dissociate all ES cells.
10. Leave the plate in the incubator for 20 min to allow any mitomycin c-treated feeder cells to reattach to the plate surface. The majority of the ES cells will remain in suspension.
11. After 20 min, gently remove the ES medium containing the ES cells and place into a sterile 1.5 mL tube. Centrifuge ES cells at 300 × *g* for 3 min, wash with 1× PBS, then process ES cell pellet for genotyping. Alternatively, ES cells can be passaged onto

clean 24-well plates with no feeder cells. This will eliminate the feeder cell population which might otherwise contaminate the final ES cell pellet for genotyping.

3.7 Genotyping of ES Cell Clones

We routinely use restriction enzyme digestion of a PCR amplicon of the mtDNA d-loop region for genotyping both xenocybrid fibroblast clones and xenocybrid ES cell clones. The primers listed were designed to amplify both *Mus musculus* and *Rattus norvegicus* mtDNA.

1. Prepare total DNA from the harvested ES cell clones using an extraction method of choice.
2. Use 20 ng of total DNA as the template in a PCR reaction using forward primer: 5′-ctc aac ata gcc gtc aag gc-3′ and reverse primer: 5′-acc aaa cct ttg tgt tta tgg g-3′ with 1.0 U *Taq* polymerase. Reaction parameters are 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; 35 cycles.
3. Check amplification of the d-loop PCR fragment by resolving a portion of the PCR reaction on a 1.5 % agarose gel. The amplicon should be approximately 450 bp in length (461 bp for *Mus musculus* mtDNA).
4. For genotyping, digest the d-loop PCR amplicon with approximately 5–10 units of restriction enzyme for 1 h at the required temperature. We use the following restriction enzymes in combination to identify the different murid species:

   ApoI: cuts *Mus musculus*, *Mus pahari*, *Rattus norvegicus*, and *Otomys irroratus* at different positions.

   StyI: cuts *Mus spretus* but not *Mus musculus*.

   *Bsu36*I: cuts *Mus musculus* but not *Mus spretus* or *Mus pahari*.

   ScaI: cuts *Mus musculus* but not *Mus caroli* or *Mus terricolor*.

   SpeI: cuts *Mus caroli* and *Mus terricolor* but not *Mus musculus*.

   DdeI: cuts *Mus musculus* and *Mus pahari* at different positions.
5. Analyse digested d-loop PCR amplicons on a 2.0 % agarose gel to determine the mtDNA genotype and the homoplasmy/heteroplasmy of the introduced murid mtDNA.

4 Notes

1. Choice of mtDNA donor cell lines. We have used primary skin fibroblasts from a number of different murid species, including *Mus spretus* and *Mus terricolor*, to create L-cell xenocybrid fibroblasts and ES xenocybrid cell lines [21].

2. We use a mouse ρ^0 mtDNA-less fibroblast cell line derived from the parental line LMTK$^-$ by exposure to ethidium bromide [22]. These ρ^0 cells are auxotrophic for uridine and pyruvate.
3. A female ES cell line is required for germline transmission of mtDNA. For these experiments we have used the CC9.3.1 ES cell line derived from a 129SvEv-*Gpi1c* embryo (A. Bradley, personal communication), which can contribute to the female germ line and produce normal fertile females [12, 24].
4. Mouse embryonic fibroblasts (MEFS) and mouse STO cells are commonly used as feeder cell lines. Derivatives, such as SNL 76/7 cells, have been engineered to express LIF, eliminating the requirement to add LIF exogenously to the ES medium.
5. Only new LMTK$^-$ cybrid fusions will survive in select medium as ρ^0 cells are auxotrophic for uridine and pyruvate while the BrdU will kill any surviving TK$^+$ mitochondrial donor fibroblasts.
6. R6G treatment of ES cells is quite toxic. Following 72 h treatment, the ES cells will die unless they are saved by successful fusion with mitochondrial cytoplast donors. ES cell mitochondrial depletion is virtually 100 %, evident by the very low percentage of heteroplasmic ES clones obtained following fusion (i.e. none of the ES cell's endogenous mtDNA remains, with 100 % of donor mtDNA present). We found that 1.0 μg/mL R6G is the optimal concentration for the CC9.3.1 ES cell line, however, this may not be the case for other lines. We recommend a titration of 0.5, 0.75, 1.0, and 1.25 μg/mL R6G for 72 h to determine the optimal concentration for each different ES line.
7. HAT selection will remove residual, nucleated xenocybrid fibroblasts due to their TK$^-$ nuclei.

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Chapter 13

Import of Fluorescent RNA into Mitochondria of Living Cells

Jaroslav Zelenka and Petr Ježek

Abstract

Methods of *in vivo* visualization and manipulation of mitochondrial genetic machinery are limited due to the need to surpass not only the cytoplasmic membrane but also two mitochondrial membranes. Here, we employ the matrix-addressing sequence of mitochondrial ribosomal 5S-rRNA (termed MAM), which is naturally imported into mammalian mitochondria, to construct an import system for *in vivo* targeting of mitochondrial (mt) DNA or mtRNA, in order to provide fluorescence hybridization of the desired sequences.

Key words 5S-rRNA, Mitochondrial DNA, Nucleic acid import into mitochondria, Fluorescent *in vivo* hybridization of mtDNA, Mitochondrial nucleoids

1 Introduction

Genetics of mitochondrial DNA (mtDNA) belongs to the least understood part of molecular biology [1–4]. Methods of manipulation with mtDNA and mitochondrial gene expression are virtually absent, though some progress is being made [5–12]. Considering *in vivo* visualization and manipulation of mtDNA, the key point is the transport across the plasma membrane and across the two mitochondrial membranes. Even for targeting the mtDNA by oligonucleotides in cultured cells, carriers containing positive charges are required, such as DQAsomes (micelles of dequalinium chloride, [12]), or the MitoPorter system based on lecithin liposomes doped with octylarginine stearate [13] or peptide nucleic acid directly conjugated with lipophilic cation [14]. Nevertheless, these carriers usually do not deliver oligonucleotide cargo directly to the mitochondrial matrix and hence are quite inefficient alone.

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It has been, however, demonstrated that mammalian 5S rRNA [15, 16] is readily imported into the mitochondrial matrix through the TIM & TOM complexes [7, 17–20]. This import is facilitated by rhodanese [21] and PNPase proteins [22–25]. Alternatively, short synthetic RNAs comprising two domains of the tRNA also exhibit high efficiency of mitochondrial import [26], and the carriers based on 5S rRNA were successfully used to balance heteroplasmy in cells with a portion of mtDNA bearing a large-scale deletion associated with Kearns Sayre Syndrome [7].

The investigation of the mitochondrial genetic apparatus has a significant dynamic component due to the presence of hundreds to thousands of copies of mtDNA dispersed through the mitochondrial network. To investigate the dynamics of such a complex system, the concept of fluorescent *in vivo* hybridization should be developed to allow direct visualization of the selected nucleic acid sequences directly in mitochondria.

We have turned our attention toward the combination of DQAsome delivery [12] with the natural mt 5S rRNA import pathway into mitochondria [7, 17–20]. We have employed the mt 5S rRNA mitochondria-addressing domain sequence portion and developed a carrier system to target mitochondrial RNA and mtDNA [27]. In addition, on the basis of such a facilitated import system, we have developed a concept of mt fluorescent *in vivo* hybridization (mtFIVH). However, the system is dependent on the accessibility of the desired sequence in the mitochondrial matrix due to the specificity of mtDNA genetics and the structure of mtDNA within mtDNA nucleoids.

2 Materials

Prepare all buffers with ultrapure water and analytical grade reagents.

1. Phosphate buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL of deionized water. Adjust pH to 7.4 with 1 M HCl. Make up to 1 L with water. Sterilize in an autoclave and store at 4 °C for up to 1 month.
2. DQAsomes stock solution: Dissolve 16 mg of dequalinium chloride in 3 mL of methanol, transfer to a 25 mL evaporation flask and evaporate on rotary evaporator at 40 °C. Resuspend the residue in 3 mL of PBS and transfer to a sterile centrifugation tube. Sonicate with an ultrasonic homogenizer with microtip until solution is clear with slight opalescence (*see* **Note 1**). Store at room temperature for up to 1 month.
3. Acetate buffer: Dissolve 18.6 g of sodium acetate and 1.41 mL of glacial acetic acid in 800 mL of water. Adjust pH to 5.6 and make up to 1 L with water. Store at 4 °C for up to 1 month.

4. Periodate solution: Dissolve 11 mg of sodium periodate in 1 L of water. Prepare fresh for each labeling (*see* **Note 2**).
5. Sulfite solution: Dissolve 26 mg of sodium sulfite in 1 L of water. Prepare fresh for each labeling (*see* **Note 2**).
6. Labeling solution: Dissolve hydrazide-modified dye in anhydrous DMSO to reach 10 mM concentration (e.g. 1 mg of AlexaFluor® 647 hydrazide in 83 μL of DMSO or 1 mg of AlexaFluor® 488 hydrazide in 170 μL of DMSO; AlexaFluor® is a trademark of Life technologies, Carlsbad, CA, USA). Store at −20 °C in dark for up to 6 months. Avoid repeated freeze/thaw cycles by creating small (5 μL) aliquots.
7. LiCl solution: Dissolve 336 mg of LiCl in 600 μL of water and make up to 1 mL with water.
8. 75 % Ethanol: Mix 7.5 mL of ethanol with 2.5 mL of water.

3 Methods

3.1 Design of the Probes

For successful import into mitochondria, each probe must be designed with mitochondria-addressing sequence (MAM) at its 5′ end and with hybridization sequence on its 3′ end. MAM sequence is derived from the γ-domain of human 5S ribosomal RNA as described in [19].

The composition of MAM sequence on RNA is:

5-GGCCTGGTTAGTACTTGGATGGGAGACCGCCAAG GAATACCGGGTG-3.

The hybridization sequence should be ~20 nucleotides long and be antisense to DNA or RNA sequence of interest (*see* **Note 3**).

3.2 In Vitro Synthesis of the Probes

If you do not use commercial custom synthesis of the probe, it can be synthesized with an *in vitro* transcription system (e.g. RiboMAX™ Large Scale RNA Production System—T7 from Promega, Madison, WI, USA).

For transcription with this system, you need to purchase two DNA oligonucleotides. First, encoding the transcription initiation sequence for T7 RNA polymerase, and second, encoding the template for your RNA probe and the antisense strand of transcription initiation sequence.

First DNA oligo: 5-TAATACGACTCACTATAGGG-3.

Second DNA oligo:

5-Sequence identical with the target mitochondrial sequence-CACCCGGTATTCCTTGGCGGTCTCCCATC-CAAGTACTAACCAGGCCCTATAGTGAGTCGTATTA-3.

Once you have these DNA oligonucleotides, you anneal them, perform *in vitro* transcription and purify resulting RNA according to the manufacturer's protocol to yield up to milligram quantities of the probe.

3.3 Labeling of the Probe

For fluorescence *in vivo* hybridization, the RNA probe should be labeled on its 3′ end with fluorescent dye. Here, we use periodate activation of the 3′ end according to [28] and labeling with AlexaFluor® hydrazide dyes (Life technologies, Carlsbad, CA, USA). This protocol yields amount of probe suitable for ten transfections:

1. Dissolve RNA probe in water to yield 10 μM solution (~22.5 μg/100 μL).
2. Transfer 100 μL of probe solution to a 2 mL microtube and add 100 μL of acetate buffer and 200 μL of periodate solution. Incubate at room temperature for 90 min.
3. Stop the reaction with 100 μL of sulfite solution and incubate at room temperature for 15 min.
4. Add 3 μL of labeling solution and incubate at 37 °C in the dark for 3 h.
5. Precipitate RNA by admixing 50 μL of LiCl solution and 1.25 mL of ethanol. Incubate at −20 °C for 3 h (or overnight).
6. Centrifuge at 15,000 × *g* for 20 min.
7. Remove the supernatant and wash/centrifuge the precipitate several times with 75 % ethanol until the supernatant is clear.
8. Allow the resulting pellet to air-dry and dissolve the residue in 10 μL of water. Store the resulting probe solution at −20 °C in the dark for up to 1 month.

3.4 Transfection of Cells

Perform the procedure in media without antibiotics and pH indicator. One day before transfection, seed the cells in complete media on coverglass slips or equivalent. The poly-l-lysination of the glass does not interfere with the assay. The cell confluence on the day of transfection should be ~50 %.

1. Dilute DQAsomes stock solution with serum-free media (1 μL of DQA per 50 μL of media per 1 cm^2 coverglass).
2. Dilute the probe solution with serum-free media (1 μL of DQA per 50 μL of media per 1 cm^2 coverglass).
3. Combine both fractions and incubate the resulting transfection solution at room temperature for 20 min.
4. Change the media in wells with coverglasses to 900 μL of serum-free media per 1 cm^2 of coverglass.
5. Add 100 μL of transfection solution per 1 cm^2 of coverglass.

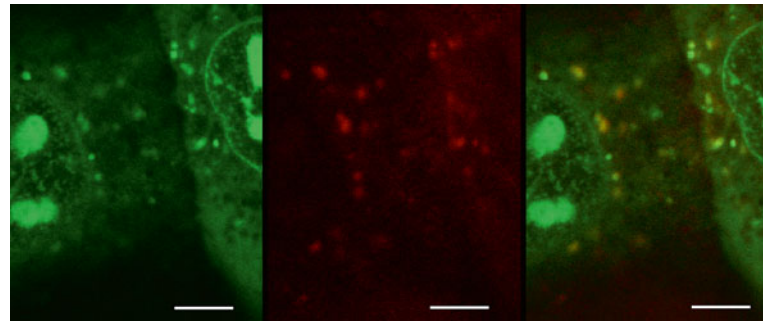

Fig. 1 Fluorescence *in vivo* hybridization of HepG2 cells stained with Syto 9 dye (*green*) to visualize mitochondrial nucleoids and nuclei. Cells were transfected as described in this protocol with probe containing MAM sequence, hybridization sequence complementary to mtND5 gene and AlexaFluor® 647 label (*red*). Bar = 5 μm

6. After 4–6 h, change the media in wells with coverglasses for complete media (*see* **Note 4**).
7. Optimal time for microscopic inspection of imported RNA may vary depending on the cell type. It is generally in the interval between immediately after changing the media to 24 h after changing the media (*see* **Note 5**). An example of cells transfected with labeled probe is shown in Fig. 1.

4 Notes

1. Dissolving of DQA could take up to 10 min and temperature of the solution may rise up to 50 °C. However, once the solution is made, it remains stable at room temperature.
2. It is worth performing sequential dilution to spare water. The given amount of salt is dissolved in 1 mL of water. Then, 100 μL of solution is transferred to a new tube with 900 μL of water. This step is repeated twice to reach 1 mL of solution with final concentration of salt.
3. The hybridization sequence should not form hairpin structures with the MAM sequence. Check this option with software for primer design.
4. DQA is slightly toxic to cells and mitochondria. Long-term treatment will result in a disintegrated mitochondrial network and round-shaped cells.
5. Import efficiency is never complete. You should use a colocalization probe, for example a marker of mitochondria (Mitotracker, tetramethylrhodamine) or mitochondrial nucleoids (Sybr green, Syto 9) for every experiment. Be sure not to over-stain the samples as the signal from the probe could be quenched.

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Part V

Purification of Proteins Involved in Mitochondrial DNA Replication and Transcription

Chapter 14

Purification and Comparative Assay of the Human Mitochondrial Replicative DNA Helicase

Fernando A. Rosado-Ruiz, Minyoung So, and Laurie S. Kaguni

Abstract

The replicative mitochondrial DNA (mtDNA) helicase is essential for mtDNA replication and maintenance of the mitochondrial genome. Despite substantial advances that have been made in its characterization, there is still much to be understood about the functional roles of its domains and its interactions with the other components of the minimal mitochondrial DNA replisome. Critical to achieving this is the ability to isolate the enzyme in a stable, active form. In this chapter we describe a modified, streamlined purification strategy for recombinant forms of the enzyme. We also present assays to assess its helix unwinding activity and the stimulatory effects of the mitochondrial single-stranded DNA-binding protein (mtSSB). Finally, we describe a concentration/buffer exchange method that we have employed to achieve greater enzyme stability and appropriate conditions for biochemical and biophysical studies.

Key words Mitochondrial DNA replication, mtDNA helicase, Helicases, Mitochondrial single-stranded DNA-binding protein, Human

1 Introduction

Since the first report of a human mitochondrial DNA helicase belonging to the DnaB family in 2000 [1], substantial advances have been made in characterizing the protein. Both the human protein, also known as Twinkle (for bacteriophage T7 gp4-like protein with intramitochondrial nucleoid localization), and its *Drosophilia* analog have shown to serve as the replicative mtDNA helicase, and are essential in animal development [1–3].

Biochemical studies with recombinant mtDNA helicase have demonstrated that it shares many properties of replicative DNA helicases [4]. It unwinds dsDNA in an NTP-dependent reaction with 5′ → 3′ polarity. Efficient DNA unwinding requires a substrate with a 5′-ssDNA loading site and a 3′-ssDNA tail, which resembles a DNA replication fork, similar to the requirements of T7 gp4 and

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E. coli DnaB [4]. As in other systems, the DNA unwinding activity of the mtDNA helicase is stimulated by its endogenous single-stranded DNA-binding protein [4–6].

Sequence and functional similarities between the mtDNA helicase and T7 gp4 have prompted studies of the architecture of the human mtDNA helicase. Like the T7 gp4, the mtDNA helicase has a modular architecture comprising two major domains, an N-terminal primase-like domain and a C-terminal helicase domain. These are separated by a flexible linker region [7]. However, despite the overall sequence similarity of the human mtDNA helicase with T7 gp4, the sequence identity between them is relatively low, with the highest variation occurring in the N-terminal domain for which the function remains unclear.

Autosomal dominant progressive external ophthalmoplegia (adPEO) has been shown to be associated with multiple mtDNA deletions, due to mutations in the mitochondrial DNA polymerase, Pol γ, and the mtDNA helicase [8]. In the case of the latter, most of the mutations have been attributed to defects in helicase activity. Recent experiments have shown that mutations in specific residues of the N-terminal domain are directly implicated in helicase function [9, 10]. Comparative sequence analysis of the N-terminal primase-related domain suggests that in many eukaryotic lineages, mtDNA helicase may have primase function, with the exception of most metazoans, which lack critical catalytic amino acids [11, 12].

Despite the substantial advances that have been made in characterizing the human mtDNA helicase, much remains to be learned. Notably, the function of the N-terminal domain of the mtDNA helicase remains elusive. To facilitate further research, we have adopted here a streamlined purification strategy that we have modified from our previous report [13]. We also present here our standard assay to assess the DNA unwinding activity of the enzyme (Fig. 1) and its stimulation by mtSSB. Finally, we describe a

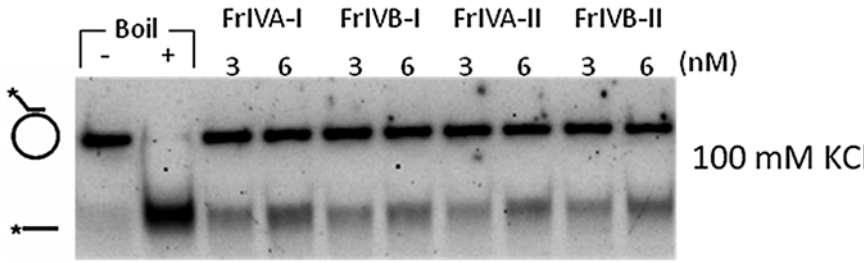

Fig. 1 Helicase unwinding activity of *Hs* mtDNA helicase. *Lane 1*: intact substrate; *lane 2*: the substrate was denatured by heating to 100 °C for 2 min. Two fractions of two independent helicase preparations were compared in the assay. The helicase concentration used is indicated above each lane. All reactions were carried out at 100 mM KCl

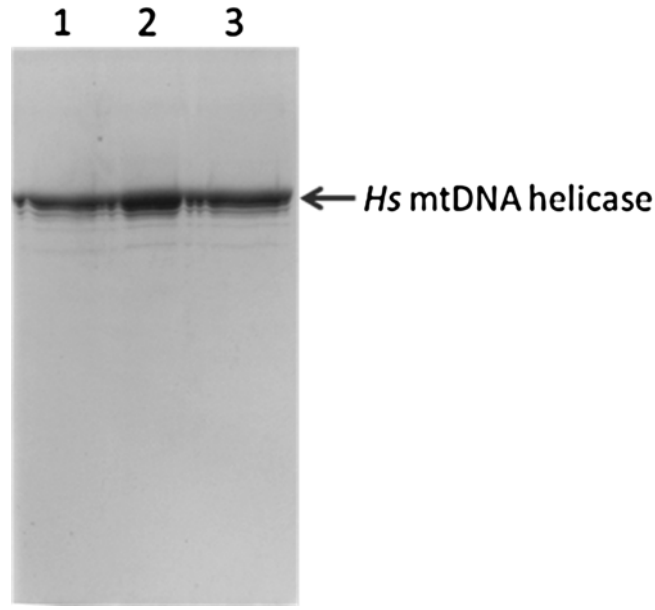

Fig. 2 Concentration and dilution of human mtDNA helicase. Protein fractions were electrophoresed in a 10 % SDS-polyacrylamide gel and stained with Coomassie Blue. *Lane 1*: *Hs* mtDNA helicase before concentration (1.2 μg); *lane 2*: *Hs* mtDNA helicase after concentration (1.2 μg); *lane 3*: *Hs* mtDNA helicase after dilution (1.2 μg)

concentration (Fig. 2) and buffer exchange strategy for the enzyme that allows versatility in conditions for general biochemical and biophysical studies.

2 Materials

2.1 Purification of Recombinant Human Mitochondrial DNA Helicase

1. 1 M Tris–HCl, pH 7.5, pH 8.0.
2. 2 M Tris–HCl, pH 6.8, pH 8.8.
3. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
4. 5 M Sodium chloride (NaCl).
5. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Aliquots stored at −20 °C.
6. 1 M Sodium metabisulfite, 1.0 M stock solutions at pH 7.5, stored at −20 °C.
7. 1 mg/mL Leupeptin prepared in 50 mM Tris–HCl, pH 7.5, stored at −20 °C.
8. 1 M Dithiothreitol (DTT), stored at −20 °C.
9. 14 M 2-Mercaptoethanol (β-Me), stored at 4 °C.

10. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4 °C.
11. 10 % Sodium dodecyl sulfate (SDS).
12. 4× Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS.
13. 4× Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
14. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
15. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at −20 °C.
16. 10 % Ammonium persulfate (APS).
17. *N,N,N′,N′*-tetramethylethylene-diamine (TEMED).
18. Insect-Xpress Protein-Free medium for insect cells with L-Glutamine (Lonza).
19. Fetal Bovine Serum.
20. Amphotericin, penicillin-G, and streptomyocin.
21. *Spodoptera frugiperda* (Sf9) cells.
22. Baculoviruses encoding N- and C-terminally His-tagged human mtDNA helicase.
23. Phosphate-buffered saline (PBS), stored at 4 °C.
24. Homogenization buffer: 25 mM Tris–HCl pH 8.0.
25. Buffer A: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol.
26. 1 M Imidazole, stored at 24 °C.
27. Nickel-nitrilotriacetic acid (Ni-NTA) agarose resin.
28. Ni-NTA column buffers:
    (a) Equilibration/Wash buffer: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 10 mM imidazole.
    (b) Elution buffer 1: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 50 mM imidazole.
    (c) Elution buffer 2: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 80 mM imidazole.
    (d) Elution buffer 3: 50 mM Tris–HCl, pH 8.0, 0.4 M NaCl, 10 % glycerol, 250 mM imidazole.
    (e) Elution buffer 4: 50 mM Tris–HCl, pH 8.0, 0.4 M NaCl, 10 % glycerol, 500 mM imidazole.
29. Heparin Sepharose (agarose) resin.
30. Heparin Sepharose column buffers:
    (a) Dilution buffer: 20 mM Tris–HCl, pH 7.5, 20 % glycerol, 0.5 mM EDTA.

(b) Equilibration buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 % glycerol, 0.5 mM EDTA.

(c) Wash buffer: 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 10 % glycerol, 0.5 mM EDTA.

(d) Elution buffer 1: 20 mM Tris–HCl, pH 7.5, 0.6 M NaCl, 10 % glycerol, 0.5 mM EDTA.

(e) Elution buffer 2: 20 mM Tris–HCl, pH 7.5, 1 M NaCl, 10 % glycerol, 0.5 mM EDTA.

31. Polyallomer tubes (14×95 mm).
32. SW 40 rotor (Beckman).
33. Glycerol gradient buffers:

(a) 1× gradient buffer: 35 mM Tris–HCl, pH 7.5, 1 mM EDTA.

(b) 12 % Gradient buffer: 35 mM Tris–HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA, 12 % glycerol.

(c) 30 % Gradient buffer: 35 mM Tris–HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA, 30 % glycerol.

2.2 Assay of Helicase Unwinding Activity

1. 60-mer oligodeoxynucleotide: (5′-$T_{(40)}$AGGTCGTTCGCTC CAAGCT-3′).
2. pBSKS+ single-stranded plasmid DNA.
3. T4 polynucleotide kinase (PNK).
4. 10× PNK buffer: 700 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT.
5. [γ-^{32}P]ATP.
6. DE-81chromatography paper cut into 1×1 cm pieces.
7. 0.3 M Ammonium formate, pH 7.5.
8. 95 % Ethanol.
9. Micro Bio-Spin P-30 Tris Chromatography column.
10. 200 Proof ethanol.
11. 1 M Tris–HCl, pH 7.5, pH 8.0.
12. 3.5 M Sodium chloride (NaCl).
13. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
14. 5× Hybridization buffer: 50 mM Tris–HCl, pH 8.0, 1.0 M NaCl, 0.15 M Na citrate, 20 mM EDTA.
15. 3 M Sodium acetate.
16. 3 M Potassium chloride.
17. 1 M Tris–HCl, pH 7.5 and pH 8.0.
18. 1 M Magnesium chloride ($MgCl_2$).
19. Bovine serum albumin.

20. 5× Helicase buffer: 50 % glycerol, 2.5 mg/mL BSA, 20 mM $MgCl_2$, 100 mM Tris–HCl pH 7.5.
21. 1 M Dithiothreitol (DTT), stored at –20 °C.
22. 0.4 M Potassium chloride (KCl).
23. 200 mM ATP, aliquots stored at –20 °C.
24. 10 % Sodium dodecyl sulfate (SDS).
25. Recombinant human mtDNA helicase.
26. 10× Stop solution: 6 % SDS, 100 mM EDTA.
27. 10× Sample buffer: 0.25 % Bromophenol blue, 50 % glycerol.
28. 30 % Polyacrylamide (59:1; acrylamide:bisacrylamide), stored at 4 °C.
29. 5× TBE buffer: 450 mM Tris–HCl-borate, 10 mM EDTA.
30. Phosphor Screen.
31. Storm 820 Scanner.

2.3 Assay of Stimulation of DNA Unwinding Activity by mtSSB

1. Recombinant human mitochondrial single-stranded DNA binding protein.
2. All other materials are as described under the materials section of the assay for helicase unwinding activity.

2.4 Concentration and Buffer Exchange of Recombinant Human Mitochondrial DNA Helicase

1. Amicon ultra centrifugal filters, 30 and 100 K cut-off.
2. 0.05 % Tween 20.
3. Milli-Q water.
4. Purified recombinant human mitochondrial DNA helicase (Fr IV) in 35 mM Tris–HCl, pH 7.5, 330 mM NaCl, 20 % glycerol.
5. Purified recombinant human mitochondrial DNA helicase (Fr III) in 50 mM Tris–HCl, pH 7.5, 600 mM NaCl, 250 mM imidazole/10 % glycerol.
6. Dilution buffer for microscopy: 35 mM Tris–HCl, pH 7.5, 330 mM NaCl.
7. Pre-incubation solution for low salt glycerol gradient: 50 mM HEPES, pH 8.0, 10 % glycerol, 4.8 mM $MgCl_2$, 2.4 mM ATPγS.
8. Glycerol gradient low salt buffers:
   12 % Gradient low salt buffer: 50 mM HEPES, pH 8.0, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM ATPγS, 0.02 % dodecyl maltoside, 12 % glycerol.
   30 % Gradient low salt buffer: 50 mM HEPES, pH 8.0, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM ATPγS, 0.02 % dodecyl maltoside, 30 % glycerol.
9. Polyallomer tubes (14×95 mm).

10. SW 40 rotor (Beckman).
11. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4 °C.
12. 10 % Sodium dodecyl sulfate (SDS).
13. 4× Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS.
14. 4× Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
15. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
16. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at −20 °C.
17. 10 % Ammonium persulfate (APS).
18. *N,N,N′,N′*-tetramethylethylene-diamine (TEMED).
19. Silver nitrate.
20. 1 M Arginine buffer: 1 M arginine, 35 mM Tris–HCl, pH 7.5.
21. Slide A-lyzer dialysis device (20 K).
22. Dialysis buffer for mass spectrometry: 10 mM NH_4HCO_3, 7 mM 2-mercaptoethanol, 12 % glycerol.

3 Methods

3.1 Purification of Recombinant Human Mitochondrial DNA Helicase

3.1.1 Sf9 Cell Growth and Protein Overexpression

1. Grow *Spodoptera frugiperda* cells (Sf9) to a cell density of 2×10^6 cells/mL at 27 °C in Insect-Xpress culture media containing 4.5 % fetal bovine serum. Dilute to a cell density of 1×10^6 cells/mL with Insect-Xpress media containing 4.5 % fetal bovine serum.
2. Infect cells with baculovirus encoding N- or C-terminally His-tagged helicase (cDNA sequence lacking the mitochondrial presequence) at a multiplicity of infection of 5 at 27 °C. Harvest 72 h post infection.
3. Pellet cells at $400 \times g$ for 5 min. Wash with equal volume of ice-cold PBS, repeat the centrifugation and discard the supernatant.
4. Resuspend and combine pellets in PBS using 0.015 volume of original culture. Spin at $400 \times g$ for 5 min, discard supernatant, freeze pellet in liquid nitrogen and store at −80 °C.

3.1.2 Preparation of Soluble Cytoplasmic Fraction (*See* **Note 1**)

1. Thaw cells on ice ≤60 min, resuspend in 1/45 volume of original cell culture in homogenization buffer. Allow to sit for 20 min on ice.
2. Homogenize cells in a Dounce homogenizer with a tight pestle using 20 strokes.

3. Adjust the homogenate to 1 M NaCl, followed by gentle inversion every 5 min for 45 min.
4. Centrifuge at 145,000 × *g* for 25 min in a 60 Ti rotor. Use buffer A to balance.
5. Pipet off and save supernatant (fraction I) in a pre-chilled beaker.

3.1.3 Nickel-Nitrilotriacetic Acid Chromatography

1. Pack and equilibrate a nickel-nitrilotriacetic acid (Ni-NTA) column (3.5 mL of resin/L of cells) with 10 column volumes (CV) of equilibration buffer at 1 CV/h.
2. Dilute fraction I with equal volume of buffer A and load onto column at a flow rate of 1–1.5 CV/h.
3. Wash column with wash buffer at flow rate of 3 CV/h, and collect 1 CV fractions.
4. Elute column with elution buffers containing 50, 80, 250, and 500 mM imidazole at 3 CV/h. Collect 1/4, 1/4, 1/6, and 1/4 CV fractions, respectively (*see* **Note 2**).
5. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining, and pool fractions accordingly (fraction II).

3.1.4 Heparin Sepharose Chromatography

1. Pack and equilibrate a heparin Sepharose column at a ratio of 10 mg of total protein in fraction II/mL of resin with equilibration buffer at a flow rate of 1 CV/h.
2. Dilute fraction II to an ionic equivalent of 150 mM NaCl. Load onto column at a flow rate of 2–3 CV/h (*see* **Note 3**).
3. Wash the column with 4 CV wash buffer at a flow rate of 3 CV/h, collecting 1/2 CV fractions.
4. Elute column with elution buffers containing 600 mM (3CV) and 1000 mM (3CV) NaCl at a flow rate of 3 CV/h, collecting 1/6 and 1/4 CV fractions, respectively (*see* **Note 4**).
5. Adjust wash and first eight fractions to 1 M NaCl to stabilize the low-salt eluted protein.
6. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining, and pool fractions accordingly (fraction III).

3.1.5 Glycerol Gradient Sedimentation

1. Prepare 12–30 % glycerol gradients in polyallomer tubes for use in a Beckman SW 40 rotor and chill on ice at least an hour before use.
2. Layer fraction III onto the gradients. Use 1× glycerol gradient buffer to adjust sample and make sure it floats atop the gradients.
3. Centrifuge at 96,600 × *g* for 40 h at 4 °C, then fractionate by collecting fractions of 200–300 μL.

4. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining and pool fractions accordingly (fraction IV).
5. Aliquot fraction IV in an appropriate volume for further studies, freeze in liquid nitrogen and store at –80 °C.
6. Analyze fraction IV by SDS-PAGE on 10 % minigels followed by Coomassie staining to determine purity and yield.

3.2 Assay of Helicase Unwinding Activity

3.2.1 5′-End Labeling of 60-mer Oligodeoxyribonucleotide

1. The kinase reaction (50 μL) contains 1× PNK buffer, [γ-^{32}P]ATP (0.66 μM, 4500 Ci/mmol), 700 pmol (as nt) of oligonucleotide, and 20 units of T4 polynucleotide kinase.
2. Incubate reaction for 30 min at 37 °C.
3. Spot 0.25 μL of the reaction onto 5 squares of 1 × 1 cm DE-81chromatography paper. Wash 3 squares in 0.3 M ammonium formate twice for 5 min, then once in 95 % ethanol. Dry all 5 squares under heat lamp for 5 min. Count radioactivity on each square in scintillation fluid, and calculate incorporation (*see* **Note 5**).
4. Purify labeled oligonucleotide using a Micro Bio-Spin P-30 Tris chromatography column.

3.2.2 Annealing the Labeled Oligonucleotide to pBSKS+ Single-Stranded Plasmid DNA

1. The hybridization reaction (60 μL) contains 4× excess pBSKS+ plasmid DNA over labeled oligonucleotide in 1× hybridization buffer.
2. Incubate at 65 °C for 60 min followed by incubation at 37 °C for 30 min. This generates a 20 bp dsDNA region with a 40 nucleotide 5′-tail.
3. Adjust reaction to a final concentration of 0.3 M sodium equivalent with 3 M sodium acetate in a final volume of 100 μL.
4. Add 2.5 volume of 200 proof ethanol and vortex to mix.
5. Incubate at –20 °C for 60 min.
6. Spin at 20,000 × *g* for 20 min and discard supernatant.
7. Quick-spin dry the pellet and air dry at room temperature for 4 min.
8. Resuspend pellet in 60 μL of 20 mM Tris–HCl, pH 7.5, 50 mM KCl, 2 mM EDTA.
9. Calculate the concentration of substrate using the concentration of the pBSKS+ plasmid DNA in the hybridization reaction (assume 100 % annealing and recovery).

3.2.3 Helicase Unwinding Activity Assay (*See* **Note 6**)

1. Prepare a master reaction mix such that each reaction contains 1× helicase buffer, 10 mM dithiothreitol, 3 mM ATP, and 20 fmol of ^{32}P-60-mer/pBSKS+ substrate. Prepare the reaction mixture in a microcentrifuge tube, vortex and centrifuge briefly.

2. Dispense the mix into microcentrifuge tube(s) on ice, adjust salt to desired concentration with addition of 0.4 M KCl and water for a final volume of 50 μL after considering the addition of desired concentration of the helicase (*see* **Note 7**).
3. Pre-incubate tube(s) at 37 °C for 10 min prior to adding the helicase.
4. Add helicase to desired concentration (in nM as hexamer) and incubate reaction tube(s) at 37 °C for 30 min.
5. Stop the reaction(s) with addition of 5 μL of 10× stop solution.
6. Add 5 μL of sample buffer, mix and centrifuge sample(s).
7. Load sample(s) onto a pre-run (600 V for 2 h) 22 % PA/TBE gel and electrophorese for 30 min at 600 V in 1× TBE until dye-front migrates to approximately 3 cm. Use a fan for cooling.
8. Dry gel under vacuum with heat and expose to a Phosphor Screen, 4 h to overnight.
9. Scan Phosphor Screen using Storm 820 scanner. Determine the volume of each band and subtract background using computer integration analysis (*see* **Note 8**).

3.3 Assay of Stimulation of DNA Unwinding Activity by mtSSB

3.3.1 Substrate Preparation for Helicase Unwinding Activity Assay

1. Substrate for this assay is prepared as described for the helicase unwinding activity assay.

3.3.2 Stimulation of DNA Unwinding Activity of Human mtDNA Helicase by mtSSB

1. Prepare a master reaction mix such that each reaction contains 1× helicase buffer, 10 mM dithiothreitol, 3 mM ATP and 20 fmol of ^{32}P-60-mer/pBSKS+ substrate. Prepare the reaction mixture in a microcentrifuge tube, vortex and centrifuge briefly.
2. Dispense the mix into microcentrifuge tube(s) on ice, adjust salt to desired concentration with addition of 0.4 M KCl and water for a final volume of 50 μL after considering the addition 3.5 nM of the helicase and the desired concentration(s) of mtSSB (*see* **Note 9**).
3. Add the desired concentration(s) of mtSSB.
4. Pre-incubate tube(s) at 37 °C for 10 min prior to adding the helicase.
5. Follow **steps 4–9** as described for the helicase unwinding activity assay.

3.4 Concentration and Buffer Exchange of Recombinant Human Mitochondrial DNA Helicase

3.4.1 Concentration

1. Fill an Amicon ultra centrifugal filter (100 K cut-off) with 0.05 % Tween 20. Incubate at room temperature for ≥1 h. Discard Tween 20 and rinse the device thoroughly with milli-Q water (*see* **Note 10**).
2. Add appropriate amount of the purified recombinant human mtDNA helicase (Fr III or Fr IV) to the Amicon ultra centrifugal filter device (*see* **Note 11**).
3. Spin the device at 2000 × *g* at 4 °C until volume of the concentrated protein reaches the desired level. Every 5 min, stop the centrifuge and gently mix the concentrated protein solution with a pipette (*see* **Notes 12** and **13**).
4. To recover the concentrated mtDNA helicase, gently mix the concentrated protein solution and pipette into a new microcentrifuge tube.

3.4.2 Dilution

This procedure aims to prepare recombinant human mtDNA helicase stably in less than 5 % (v/v) glycerol for physical studies such as cryo-electron microscopy. All steps should be performed on ice.

1. The purified recombinant human mtDNA helicase should be concentrated to at least 0.5 mg/mL by using the method described above prior to decreasing glycerol amount.
2. Dilute the protein in 4 volumes of dilution buffer for microscopy. Add the buffer drop-wise to the protein solution with occasional tapping (*see* **Note 14**).

3.4.3 Desalting

This procedure aims to prepare recombinant human mtDNA helicase at near-physiological ionic strength.

1. Prepare a 12–30 % glycerol gradient with glycerol gradient low salt buffers in polyallomer tubes (14 × 95 mm), and chill in the cold room at least 1 h (*see* **Note 15**).
2. Preincubate 250 μL (total protein amount: 0.1–1 mg) of the purified recombinant human mtDNA helicase Fr III with 1.25 mL of pre-incubation buffer at 21 °C for 5 min. (Final concentrations should be 10 % glycerol, 4 mM $MgCl_2$, 2 mM ATPγS, and 100 mM NaCl) (*see* **Note 16**).
3. Layer the protein on the top of the gradient.
4. Centrifuge at 137,500 × *g* for 16 h at 17 °C in SW 40 rotor (*see* **Note 17**).
5. Fractionate by collecting 300 μL fractions. Analyze 12–15 μL per fraction by SDS-PAGE on 10 % gels and stain them with silver nitrate. Pool the peak fractions.
6. Concentrate the gradient pool with an Amicon ultra centrifugal filter (30 K cut-off) above. To stabilize the mtDNA helicase under physiological ionic strength during concentration,

add 1 M arginine buffer at 1/10 volume of the gradient pool (the final concentration of arginine should be 100 mM) (*see* **Note 18**).

7. Aliquot the concentrated mtDNA helicase and freeze in liquid nitrogen. Store at –80 °C.

3.4.4 Dialysis

This procedure aims to prepare recombinant human mtDNA helicase in a buffer for Q-TOF mass spectrometry.

1. The purified recombinant human mtDNA helicase should be concentrated to at least 0.5 mg/mL using the method in Subheading 3.4.1 prior to change the buffer by dialysis.
2. Add 0.5–1 mg of the protein to a Slide A-lyzer dialysis device (20 K cut-off).
3. Dialyze the protein in dialysis buffer for mass spectrometry at 100 times the volume of sample at room temperature. Replace the buffer every 30 min for 3 h (*see* **Note 19**).
4. Recover the dialyzed protein from the device with a syringe.
5. Spin the recovered protein at 10,000 × *g* for 10 min. Transfer supernatant to a new microcentrifuge tube in order to use for mass spectrometry (*see* **Note 20**).

4 Notes

1. All buffers contain 1 mM PMSF, 10 mM sodium metabisulfite, 2 μg/mL leupeptin, and 10 mM 2-mercaptoethanol. All steps are to be performed at 4 °C.
2. Helicase protein elutes in fractions corresponding to the buffer containing 250 mM imidazole.
3. Precipitate will form as ionic strength decreases to 150 mM NaCl. As sample loads onto the column this precipitate will accumulate on the resin bed. Gently swirl fraction II every 30 min while loading.
4. As ionic strength increases precipitate will go back into solution.
5. To calculate incorporation: [(average cpm of three washed papers)/(average cpm/pmol of unwashed papers)]/pmol ends in the reaction.
6. Set aside two reactions prior to addition of enzyme to be used as controls. One should be boiled for 2 min at 100 °C to serve as the positive control, and the other remains unboiled to serve as the negative control.
7. Take into consideration the salt contribution of the helicase.

8. DNA unwinding is defined as the fraction of radiolabeled DNA species that is single-stranded: % unwinding = $(V_P/(V_S + V_p)) \times 100$; V_P represents the volume of the product and V_S represents the unreacted substrate in the lane of interest.
9. Take into consideration the salt contribution of both the helicase and the mtSSB.
10. Treatment with 0.05 % Tween 20 reduces non-specific binding of human mtDNA helicase to the membrane in the device.
11. Because the mtDNA helicase is likely to precipitate during centrifugal concentration, the protein should be in a buffer with at least 300 mM NaCl.
12. Mixing is required in order to disrupt the protein concentration gradient inside the centrifugal filter device because highly concentrated mtDNA helicase (more than 2 mg/mL) is likely to precipitate.
13. Do not touch the membrane while mixing the concentrated protein.
14. Make sure that the dilution buffer has at least 300 mM NaCl to avoid precipitation of the recombinant mtDNA helicase.
15. Cofactors ($MgCl_2$ and ATPγS) and detergent (dodecyl maltoside) are required in the low salt dilution buffer to improve the solubility of the mtDNA helicase at physiological ionic strength [14].
16. Fraction IV of the human mtDNA helicase purification can also be used. In this case, pre-incubation buffer should be prepared differently to achieve the same final concentrations of glycerol, cofactors and salt as for fraction III.
17. The mtDNA helicase is more soluble at 17 °C than at 4 °C [14].
18. 100 mM arginine improves the solubility of the human mtDNA helicase in solutions with ≤10 % glycerol.
19. 2-Mercaptoethanol should be added to the dialysis buffer just before use.
20. Precipitate will form because ionic strength decreases during the dialysis.

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Chapter 15

Expression and Purification of Mitochondrial RNA Polymerase and Transcription Factor A from *Drosophila melanogaster*

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Abstract

Mitochondrial gene expression is essential in all organisms. Our understanding of mitochondrial transcription on a biochemical level has been limited by the inability to purify the individual protein components involved in mitochondrial gene expression. Recently, new systems have been identified that permit purification of these proteins from bacteria. However, the generalizability of these systems is not clear. Here, we have applied the technology from the Cameron lab to express and purify mitochondrial RNA polymerase and transcription factor A from *Drosophila melanogaster*. We show that the use of SUMO system to produce SUMO fusion proteins in bacteria is effective not only for the human and mouse proteins, but also for the fly proteins. The application of this system to produce the mitochondrial proteins from other organisms should permit detailed understanding of mitochondrial transcription from any organism.

Key words Mitochondria, Mitochondrial transcription, Transcription factor A, Mitochondrial RNApolymerase, Protein expression, Protein purification

1 Introduction

It is becoming increasingly clear that the ability to understand the regulation of mitochondrial gene expression requires the ability to study transcription reconstituted from purified components *in vitro*. For example, mitochondrial transcription factor A (TFAM) is now considered to be a key regulator of mitochondrial transcription [1–6]. The ability to illustrate its role in mitochondrial transcriptional control required the use of transcription systems reconstituted from purified components rather than cell-free extracts [5]. Until recently, the purification of mitochondrial proteins has been achieved only using baculovirus systems, and often required co-expression of proteins such as mitochondrial RNA polymerase and transcription factor B2 [4, 7]. However, the efforts

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by the Cameron and Temiakov labs have simplified this situation [5, 8–11]. To determine the breadth of the applicability of the SUMO technology published by the Cameron laboratory [12] to the production of mitochondrial transcription proteins, we have extended it to purify mitochondrial RNA polymerase (*Dm-mtRNAP*) and transcription factor A (*Dm*-TFAM) from *Drosophila melanogaster*. The SUMO system results in the production of SUMO fusion proteins that can be purified by using an N-terminal, six-histidine tag and subsequently cleaved by a SUMO protease (Ulp1) to produce the "authentic" untagged proteins [12]. In addition, both the expression and the yield of the SUMO fusion proteins are enhanced by employing the Overnight Express AutoInduction System that allows for the expression of proteins in bacteria at high cell density, without the need to monitor cell density and the timely addition of an exogenous inducer such as IPTG. Here we report the expression and the purification of *Dm-mtRNAP* and *Dm*-TFAM, and the surprising observation that *Dm*-TFAM alone can activate transcription on a fragment containing *Drosophila* mtDNA sequence.

2 Materials

2.1 Protein Expression by Auto-Induction

1. Equipment: 37 °C heat block, microcentrifuge, 30 °C incubator, refrigerated incubator shaker, visible spectrophotometer, 4 °C refrigerator or cold box, centrifuge, centrifuge bottles, gel electrophoresis equipment, power supply.
2. pSUMO expression plasmid (Life Sensors) containing cloned mitochondrial transcription genes for *Dm*-mtRNAP and *Dm-TFAM* from *Drosophila melanogaster*. NCBI accession numbers NM_134721.3 and NM_079691.3 for *Dm*-mtRNAP and *Dm*-TFAM, respectively. Expression plasmids resulted in the production of *Dm*-mtRNAP (amino acids 77–1369) and Dm-TFAM (amino acids 1–257) (*see* **Note 1**).
3. BL21(DE3) competent cells.
4. SOC media: 0.5 % yeast extract, 2 % tryptone, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl, autoclave sterilize.
5. NZCYM (Amresco) agar plates supplemented with 25 μg/mL kanamycin.
6. Overnight Express AutoInduction System (EMD Millipore).
7. 25 mg/mL kanamycin solution: 250 mg kanamycin in 10 mL ddH_2O, filter sterilized.
8. 2 L Erlenmeyer flasks.
9. $T_{10}E_1$: 10 mM Tris pH 8.0, 1 mM EDTA.

10. 2× SDS-PAGE sample buffer: 225 mM Tris pH 6.8, 50 % glycerol, 0.70 M β-mercaptoethanol, 0.05 % bromophenol blue, 5 % SDS.
11. 1× SDS-PAGE sample buffer: 2× SDS-PAGE sample buffer diluted with an equal volume of $T_{10}E_1$.
12. 8 and 15 % SDS-PAGE gels.
13. 1× SDS-PAGE running buffer: 25 mM Tris, 190 mM glycine, 0.1 % SDS.
14. Coomassie blue stain: 0.1 % Coomassie brilliant blue R-250, 40 % ethanol, 10 % acetic acid.
15. Destain: 10 % ethanol, 10 % acetic acid.

2.2 Protein Purification

1. Equipment: Homogenizer, French press and pressure cell, peristaltic pump and tubing, HisPur (Thermo Scientific) cobalt resin, Kontes-flex columns (10 mm × 50 mm), centrifuge, conical tubes, dialysis tubing: 3000 and 30,000 molecular weight cut-off (MWCO), gel electrophoresis equipment, stir plate, Vivaspin concentrators: 3000 and 30,000 MWCO.
2. Lysis buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, Roche Complete protease inhibitor tablets.
3. Post-lysis supplement reagents: 100 mM phenylmethylsulfonylfluoride (PMSF) prepared in ethanol, 10 % nonidet P-40 (NP-40).
4. 1st Column wash buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF, 0.1 % NP-40, Roche Complete protease inhibitor tablets (1 tablet per 50 mL wash buffer).
5. 2nd Column wash buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF.
6. Column elution buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 150 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF.
7. Ulp1 SUMO protease (Life Sensors).
8. Dialysis buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 5 mM β-mercaptoethanol.
9. Bradford reagent: 50 mg Coomassie Blue G250 dissolved in 50 mL methanol, 100 mL of 85 % H_3PO_4 was added, followed by H_2O to 1 L.

2.3 Mitochondrial Transcription Assay

1. Equipment: thermocycler, agarose gel electrophoresis equipment (gel tray, caster, comb, running apparatus), Nanodrop spectrophotometer, radioactive work space, heat blocks at 20, 32, 50, and 65 °C, large gel electrophoresis equipment (gel stand, plates, combs, and spacers), power supply, phosphorimager screen, and Typhoon variable mode imager (GE).
2. Plasmid containing cloned mitochondrial DNA promoter sequences. NCBI accession number NC_001709.1 for *Drosophila melanogaster* mitochondrion, complete genome. Two sequences that correspond to the central and rightward regions of the A+T-rich non-coding region of *Drosophila melanogaster* mtDNA (Oregon R strain) anticipated to contain promoters for both DNA strands and spanning regions 16972–17597 (designated OR-Ori) and 19108 to 19517/1–225 (designated OR-Rend) were cloned into pCR4-TOPO plasmid DNA.
3. 10× Thermopol buffer (NEB).
4. 100 mM $MgSO_4$.
5. 10× dNTPs (4:1): 4 mM dATP, 4 mM dTTP, 1 mM dCTP, 1 mM dGTP, dilute each dNTP in ddH_2O from 100 mM Ultrapure dNTPs solution.
6. DNA oligonucleotide primers: M13 forward and M13 reverse.
7. Deep Vent DNA polymerase (NEB).
8. 5× Xylene cyanol (XC) solution: 50 % glycerol, 2.5× TBE, 0.05 % xylene cyanol.
9. 1.0 % Agarose gel in 0.5× TBE with 0.25 μg/mL ethidium bromide.
10. 0.5× TBE: 44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA with 0.25 μg/mL ethidium bromide.
11. Wizard SV Gel and PCR Clean-Up System (Promega).
12. $T_{10}E_{0.1}$: 10 mM Tris pH 8.0, 0.1 mM EDTA.
13. 100 mM TCEP (Tris(2-carboxyethyl)-Phosphine Hydrochloride): 286 mg TCEP-HCl, 500 μL H_2O, 300 μL 10 N NaOH, check pH with pH strips and pH to 7.0 with 10 N NaOH, add H_2O to 1 mL final volume.
14. 10× Reaction buffer: 500 mM Hepes pH 7.5, 100 mM $MgCl_2$, 10 mM TCEP pH 7.0.
15. 10× BSA: 10 mg/mL diluted from 100× BSA.
16. Enzyme dilution buffer (EDB): 10 mM Hepes pH 7.5, 20 % glycerol, 2 mM TCEP pH 7.0.
17. 5 M NaCl.
18. 100 mM Ultrapure NTP solutions: ATP, CTP, GTP, UTP.
19. 10 μCi/μL [α-^{32}P]-UTP.

20. RQ1 DNase (Promega).
21. 0.2 M EDTA/4.2 M urea in 1× TBE.
22. Proteinase K.
23. Gel loading dye: 95 % formamide, 0.025 % XC, 0.025 % BPB.
24. 10 and 100 bp DNA ladder.
25. 10 μCi/μL [γ-^{32}P]-ATP.
26. Denaturing polyacrylamide gel: 6 % acrylamide/0.5 % bis-acrylamide, 42 % formamide, 7 M urea, 1× TBE.
27. Gel running buffer/1× TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

3 Methods

3.1 Protein Expression by Auto-Induction

1. Dilute pSUMO expression plasmid to 5 ng/μL in ddH_2O and add 5 μL to the bottom of sterile 1.7 mL microcentrifuge tube (*see* **Note 2**).
2. Thaw BL21(DE3) competent cells at the bench-top and add 100 μL competent cells to pSUMO plasmid DNA in the 1.7 mL tube. Place tube on ice for 10 min.
3. Transfer tube to 37 °C water bath and incubate for 1 min.
4. Place tube back on ice for 2 min.
5. Add 900 μL SOC media and transfer tube to 37 °C water bath and incubate for 60 min.
6. Place tube in microcentrifuge and spin at 16,000 × *g* for 30 s.
7. Remove the majority of the supernatant without disrupting pellet and leaving behind approximately 100 μL SOC media. Use this remaining SOC media to suspend pellet.
8. Transfer 100 μL suspended pellet to NZCYM Agar plate and spread evenly over plate.
9. Place plate in 30 °C incubator overnight, approximately 16–18 h (*see* **Note 3**).
10. Prepare 500 mL Overnight Express AutoInduction media in 2 L Erlenmeyer flask per manufacturer's instructions.
11. Add kanamycin solution to media to a final concentration of 25 μg/mL.
12. Inoculate media with a smear of colonies from transformed cells on agar plate.
13. Place flask in 37 °C incubator shaker and shake at 250 rpm until an $OD_{600} = 1.0$ as measured using visible spectrophotometer.
14. Save 1 mL uninduced sample to be prepared for SDS-PAGE (**step 18** below).

15. Once culture reaches $OD_{600} = 1.0$, place flask in 4 °C refrigerator or cold box until temperature reaches 25 °C. Alternatively, culture can be cooled directly in 25 °C incubator or at the bench-top.
16. Place flask in 25 °C incubator shaker and shake at 250 rpm for 16–20 h. The OD_{600} should reach between 10 and 20 (*see* **Notes 4** and **5**).
17. Remove 0.2 mL induced culture and prepare sample for SDS-PAGE (**step 18** below).
18. Spin uninduced and induced culture in microcentrifuge at 12,000 × *g* for 1 min and remove media.
19. Suspend pellet to an OD_{600} of 20 OD/mL in 1× SDS-PAGE sample buffer. Heat sample to 100 °C for 10 min.
20. Run 10 μL of uninduced and induced SDS-PAGE samples on the appropriate percentage of SDS-PAGE gels to confirm expression.
21. Once expression is confirmed, harvest cells by centrifugation at 6000 × *g* for 10 min at 4 °C, pour off media.
22. Suspend pellet with ice-cold $T_{10}E_1$ (use 50 mL TE per 500 mL culture).
23. Spin cells at 6000 × *g* for 10 min at 4 °C and remove TE wash.
24. Weigh cell pellet and freeze at −80 °C until use.

3.2 Protein Purification

1. Pre-equilibrate 1.0–2.0 mL cobalt resin with 10 column volumes of lysis buffer in the Kontes column by gravity or using peristaltic pump at 1 mL/min.
2. Suspend frozen cell pellets on ice with 5 mL lysis buffer per gram of cell mass.
3. Dounce homogenize cells on ice to break clumps and attain a thick suspension of bacteria cells.
4. Use ice-cold French pressure cell and lyse cells three times at 20,000 psi (internal) in the appropriate volume pressure cell (*see* **Note 6**).
5. Clarify lysate by centrifugation at 75,000 × *g* for 30 min at 4 °C. Decant and combine clarified lysate into a larger centrifuge tube.
6. Add pre-equilibrated cobalt resin to clarified lysate in centrifuge tube and swirl immediately to bind His-SUMO tagged protein to resin via batch binding. Rotate capped tube for 30 min to bind His-tagged protein to resin (*see* **Note 7**).
7. Gently sediment loose cobalt resin by centrifugation at 200–300 × *g* for 2 min at 4 °C. Rotate the centrifuge tube to 180° and repeat process until all resin is at the bottom of tube.

Decant supernatant, which is the "flow through" or "pass" to a new beaker. Suspend resin in several mL of 1st column wash buffer, transfer resin to chromatography column and gravity pack, collecting the remaining flow through (*see* **Note 8**).

8. Wash resin with 10 column volumes of 1st column wash buffer containing the full complement of protease inhibitors and NP-40 (*see* **Note 9**).
9. Wash resin with 10 column volumes of 2nd column wash buffer containing only pepstatin A, leupeptin, and PMSF (no Roche tablets or NP-40). The column should be washed to near baseline as measured by using the Bradford assay.
10. Elute protein with elution buffer, collect 1-column volume fractions. Use the Bradford assay to determine location of protein followed by SDS-PAGE to determine purity. Mix 5μl-10μl of each fraction with an equal volume of 2× SDS-PAGE sample buffer, heat to 100 °C for 10 min and load SDS-PAGE gel.
11. Pool elution fractions based upon purity. Measure protein concentration by using the Bradford assay.
12. Add 1 μg Ulp1 per 1 mg purified SUMO fusion protein to cleave SUMO from protein of interest (*see* **Note 10**). Mix well and transfer protein solution to dialysis tubing. Use 3000 MWCO for TFAM, and 30,000 MWCO for *Dm-mtRNAP*. Dialyze overnight for 16–18 h with stirring.
13. Next morning break dialysis and transfer protein to a fresh conical tube. Check sample via SDS-PAGE for completion of cleavage reaction (Fig. 1).
14. Pre-equilibrate 0.5 mL cobalt resin in the Kontes column with 10 column volumes of dialysis buffer.
15. Pass entire volume of dialyzed, cleaved protein over cobalt resin to bind SUMO, Ulp1, and any uncleaved target protein (*see* **Note 11**). A final bed volume or column volume of buffer can be used to clear resin of protein. Save pass-through as it contains final purified protein. Confirm by Bradford assay and SDS-PAGE (Fig. 2).
16. Concentrate sample as necessary using Vivaspin concentrator as per manufacturer's instructions at 4 °C. 3000 MWCO for TFAM and 30,000 MWCO for *Dm*-mtRNAP. Calculate final concentration by Bradford assay. Aliquot proteins, flash freeze on dry ice and store at −80 °C.

3.3 Mitochondrial Transcription Assay

1. Set-up 500 μL PCR reaction with 1× Thermopol buffer, 1× dNTPs (4:1 ratio), 0.5 μM DNA oligonucleotide forward and reverse primers, 0.5 ng/μL plasmid DNA containing cloned mitochondrial DNA sequences (*see* **Note 12**), 0.02 units/μL Deep Vent DNA polymerase (final reaction concentrations). Assemble in PCR tubes (*see* **Note 13**).

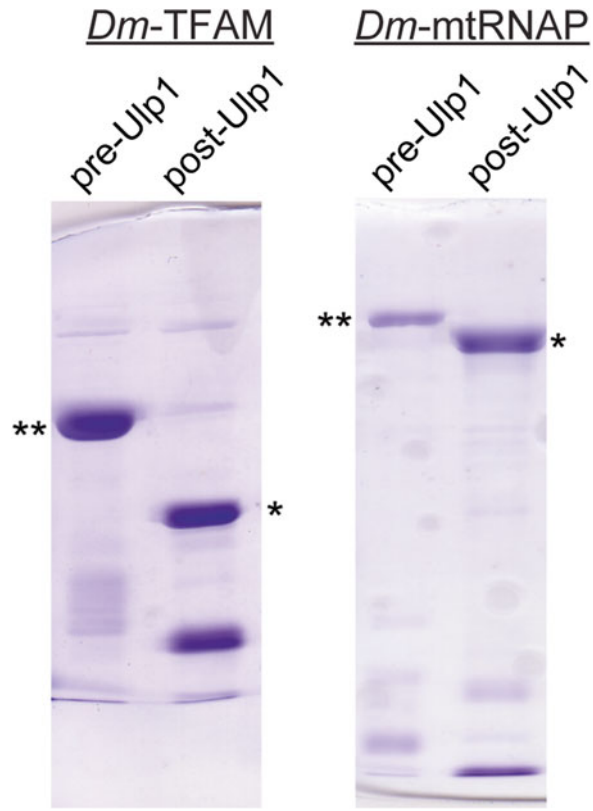

Fig. 1 Coomassie-stained SDS-PAGE gels of *Drosophila melanogaster* mitochondrial transcription proteins cleaved from the SUMO tag by the Ulp1 SUMO protease. Shown are the pre- and post-Ulp1 SUMO-fusion proteins for *Dm*-TFAM and *Dm-mtRNAP*. The ** indicates SUMO-fusion protein and * indicates cleaved target protein

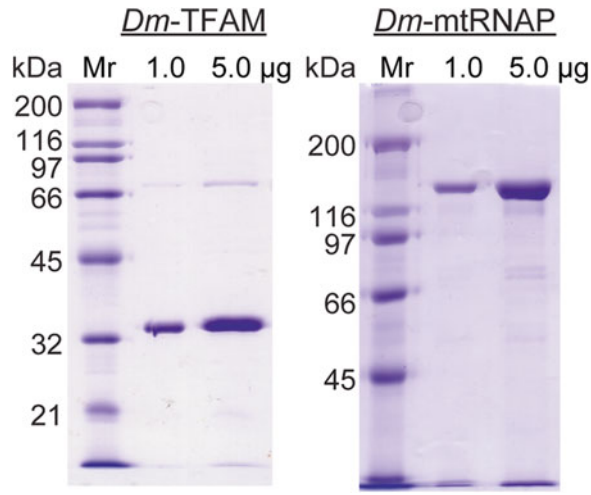

Fig. 2 Final fractions of purified mitochondrial transcription proteins. Shown are 1.0 and 5.0 µg of the purified *Dm*-TFAM and *Dm*-mtRNAP on a Coomassie-stained SDS-PAGE gel, 15 % for *Dm*-TFAM and 8 % for *Dm*-mtRNAP

2. Place PCR reactions in thermocycler with following settings: 95 °C 4 min for 1 cycle, 95 °C 15 s, 50 °C 30 s, 60 °C 3 min per 1 kb for 30 cycles, 60 °C 20 min for 1 cycle.
3. After PCR reaction finishes, mix 10 μL PCR reaction with 2 μL 5× BPB and run on 1.0 % agarose gel to verify PCR product.
4. Once PCR reaction is confirmed, purify PCR product using Wizard SV Gel and PCR Clean-Up System (follow manufacturer's instructions).
5. Quantitate PCR product using Nanodrop spectrophotometer.
6. Assemble mitochondrial transcription assay reagents at room temperature (final concentrations, 50 μL volume per each reaction): 1× reaction buffer, 1× BSA, 100 nM PCR product, 100 mM NaCl, 500 μM ATP, 500 μM CTP, 500 μM GTP, 100 μM UTP, 0.2 μCi/μL [α-^{32}P]-UTP.
7. Thaw purified mitochondrial transcription proteins on ice. Dilute *Dm*-mtRNAP to 10 μM final using ice-cold EDB. Dilute *Dm*-TFAM to 5, 10, and 25 μM final using ice-cold EDB.
8. Place mitochondrial transcription reaction mix at 20 °C for 5 min, follow subsequent steps (schematic of assay illustrated in Fig. 3a).
9. Add 1/10th volume of each *Dm*-TFAM dilution to individual transcription reactions. Wait 1 min (*see* **Note 14**).
10. Add *Dm*-mtRNAP to 1 μM final to transcription reaction to initiate reaction. Let reaction proceed for 30 min (*see* **Note 15**).
11. After 30 min, add 1 unit RQ1 DNase to each reaction. Transfer reactions to 32 °C and incubate for 10 min (*see* **Note 16**).
12. Add 1/4th volume of 0.2 M EDTA/4.2 M urea in 1× TBE to each reaction.
13. Add Proteinase K to 0.05 mg/mL final and incubate at 50 °C for 15 min.
14. Add an equal volume of gel loading dye solution to reaction, vortex, quick spin in microcentrifuge (*see* **Note 17**).
15. Label DNA ladder with [γ-^{32}P]-ATP as per manufacturer's instructions.
16. Heat reactions at 65 °C for 5 min and load 5 μL on 6 % denaturing PAGE. Run gel at 90 W for 2 h.
17. Disassemble gel and expose gel to phosphorimager screen for 10–60 min (*see* **Note 18**).
18. Scan screen using Typhoon variable mode imager (Fig. 3b).

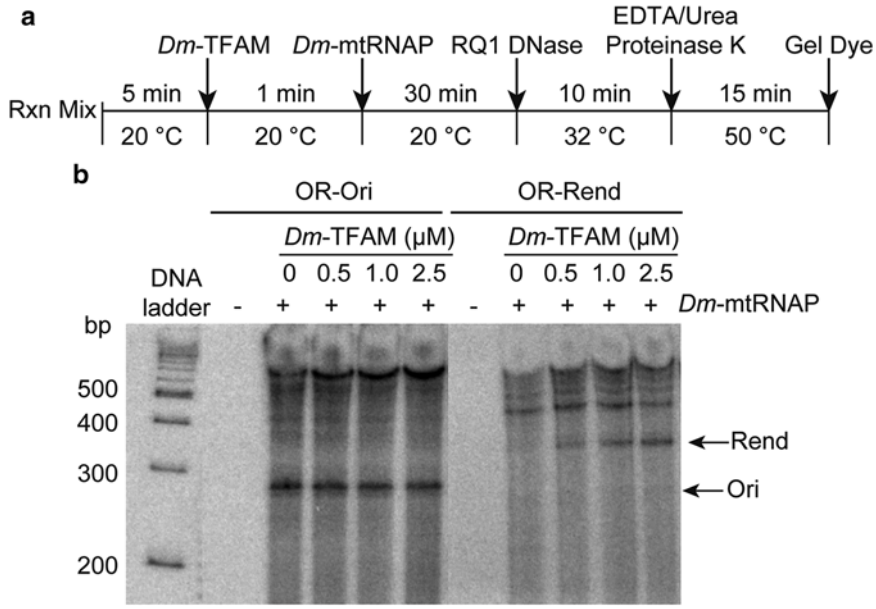

Fig. 3 Mitochondrial transcription assay. (**a**) Schematic of transcription assay. (**b**) Denaturing PAGE gel of *in vitro* mitochondrial transcription products using OR-Ori and OR-Rend as DNA templates. Ori and Rend correspond to the central and rightward regions of the A+T-rich non-coding region of *Drosophila melanogaster* mtDNA (Oregon R strain), and encompass nucleotides 16972–17597 and 19108–19517/1–225. The *arrows* indicate specific transcription products produced as a result of internal initiation using the indicated DNA fragment

4 Notes

1. Mitochondrial transcription genes are cloned into the pSUMO expression plasmid in frame with SUMO to produce a SUMO fusion protein containing a N-terminal histidine tag for affinity purification. The gene for mitochondrial RNA polymerase was cloned lacking the putative coding sequence for the mitochondrial targeting signals, and that for TFAM was cloned intact.
2. Use autoclaved sterile tips and tubes during transformation.
3. Incubate plates at 30 °C overnight, if 37 °C is used the colonies can grow too large and complicate protein expression.
4. Expression is typically induced once culture reaches an OD_{600} greater than 7.0.
5. The temperature for expression can be adjusted to maintain solubility. Typical temperatures for expression are 25 °C or 15 °C. Growth at 25 °C is performed for 16–20 h, whereas growth at 15 °C is performed for 36–40 h.
6. Lysis by French press is necessary as to not shear chromosomal DNA. Other methods may be used but could complicate purification with contaminating nucleic acid.

7. Batch binding allows for more efficient interaction with the cobalt resin for affinity purification.
8. Directly adding the resin to the Kontes column after batch binding can be performed without centrifuging sample to pellet resin.
9. It is crucial to wash the resin substantially to remove contaminants.
10. A ratio greater than 1 μg Ulp1 per mg of purified SUMO-fusion protein may be necessary to cleave completely the fusion protein to remove the SUMO tag. This can result from the nature of the terminal amino acid residue next to the cleavage site.
11. Passing the cleaved fusion protein over a second cobalt resin is necessary to remove the histidine tagged SUMO, Ulp1 and any uncleaved SUMO-fusion protein.
12. Mitochondrial DNA promoter sequences should be cloned into plasmids such as pCR4-TOPO or pUC18 such that promoter sequences can be PCR-amplified using M13 forward and reverse primers.
13. PCR reactions and cycling conditions to amplify mitochondrial DNA sequences are optimized based upon high AT-rich DNA sequences of the *Drosophila melanogaster* mtDNA genome. If mitochondrial DNA sequences to be used contain a lower A+T content, then 4 mM of each dNTP can be used in the 10× dNTP stock, and PCR cycling conditions can be varied for annealing and extension times and/or temperature. Titration of $MgSO_4$ may be required to optimize PCR reaction conditions.
14. TFAM titrations in mitochondrial transcription assays are required to determine the optimal concentration for promoter-dependent transcriptional activity.
15. Reaction times for transcription assays can vary and time points can be processed and quenched accordingly.
16. RQ1 DNase treatment is required to prevent RNA transcripts from reannealing to DNA templates during gel electrophoresis. Proteinase K treatment prevents proteins from associating with RNA transcript products in case denaturation is not complete.
17. Transcription assays contain significant amounts of radioactivity so proper care should be taken to shield user from exposure, and the user should check for contamination regularly.
18. Denaturing PAGE gel can be placed onto a suitable plastic backing and covered in plastic wrap to prevent contamination to Phosphorimager screen.

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Chapter 16

Purification and Comparative Assay of Human Mitochondrial Single-Stranded DNA-Binding Protein

Grzegorz L. Ciesielski, Fernando A. Rosado-Ruiz, and Laurie S. Kaguni

Abstract

The mitochondrial single-stranded DNA-binding protein (mtSSB) coordinates the function of replisome components at the mitochondrial replication fork. In recent years, it has been demonstrated that mtSSB stimulates the activities of DNA polymerase γ (Pol γ) and mitochondrial DNA (mtDNA) helicase in a concentration-dependent manner. Here we present a new approach to purify the human mtSSB and our standard assays to evaluate its biochemical properties, including a Gel Mobility Shift Assay (GMSA) to assess single-stranded DNA (ssDNA) binding activity, and an assay to assess SSB stimulation of Pol γ activity.

Key words Mitochondrial DNA replication, Mitochondrial single-stranded DNA-binding protein, DNA polymerase γ, Human

1 Introduction

In vitro studies suggest that three proteins, Pol γ, the mitochondrial replicative DNA helicase (also known as Twinkle), and mtSSB are the key components of the mitochondrial DNA replisome [1, 2]. They are responsible for mtDNA synthesis and proofreading, unwinding of double-stranded DNA (dsDNA), and protection of ssDNA, respectively. In addition, mtSSB has been shown to coordinate the functions and stimulate the activities of Pol γ and mtDNA helicase [3–8]. mtSSBs are 13–16 kDa proteins that function as homotetramers [3, 9]. *In vivo*, depletion of mtSSB protein causes a reduction of mtDNA copy number in cultured cells [10]. Moreover, absence of mtSSB in *D. melanogaster* is lethal at the third larval stage of development [11]. Unlike Pol γ and mtDNA helicase, no mtSSB mutations have yet been associated with human diseases, which may imply that dysfunction of mtSSB is lethal for humans at an early stage of development.

*These authors contributed equally to this work.

Matthew McKenzie (ed.), *Mitochondrial DNA: Methods and Protocols*, Methods in Molecular Biology, vol. 1351,
DOI 10.1007/978-1-4939-3040-1_16,

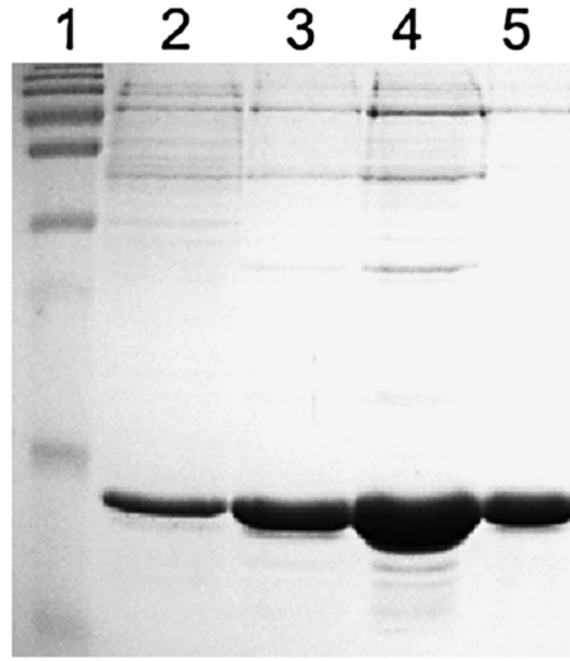

Fig. 1 Purification of *Hs*mtSSB. Protein fractions were denatured and electrophoresed in a 15 % SDS-polyacrylamide gel, and the proteins were detected by staining with Coomassie Blue. *Lane 1*: molecular weight standard; *lane 2*: soluble cell extract (Fraction I, 2.3 μg); *lane 3*: Blue Sepharose pool (Fraction II, 4.7 μg); *lane 4*: concentrated Fraction II (Fraction IIb, 8.6 μg); *lane 5*: glycerol gradient pool (Fraction III, 4.2 μg)

Our group has developed and published protocols to overexpress efficiently mtSSB variants in *E. coli* cell cultures, and to purify the target protein to near-homogeneity, which has enabled the study of various recombinant mtSSB variants of human and fruit fly origin [3–5, 7, 9, 12]. Here, we present an updated purification scheme to obtain near-homogenous recombinant human mtSSB in two key steps: affinity chromatography using Blue Sepharose and either a cation exchange chromatography using phosphocellulose, and/or sedimentation in a glycerol gradient (Fig. 1). Some mtSSB variants bind tightly to the Blue Sepharose column and cannot be eluted using NaCl. A sodium thiocyanate (NaSCN) elution is used instead, which takes advantage of the ability of SSB proteins to retain their functional conformation after denaturation and subsequent renaturation [13–16]. Inclusion of the phosphocellulose chromatography step serves both to purify further the protein, and to ensure the removal of a dsDNA-unwinding contaminant [9].

We present a GMSA as a method to assess the ssDNA-binding activity of mtSSB [7]. In this assay, we employ a 48-mer ssDNA oligonucleotide that is close to the binding-site size previously determined for human mtSSB (Fig. 2) [17, 18]. Given that mtSSB

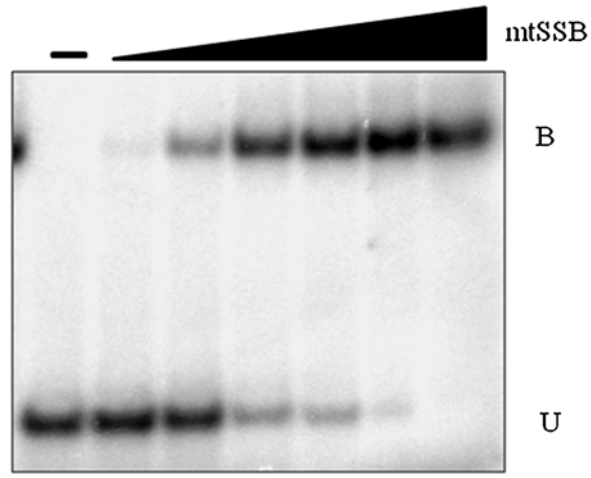

Fig. 2 GMSA of *Hs*mtSSB. Concentrations of 1, 2, 4, 6, 10, and 15 nM (as tetramer) of *Hs* mtSSB were incubated with a radiolabeled 48-mer oligonucleotide in buffer containing 50 mM NaCl. "–" denotes no added mtSSB, U and B denote the unbound and mtSSB-bound fractions, respectively

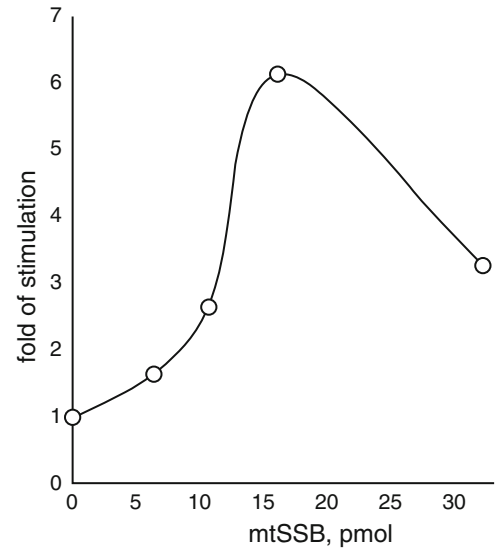

Fig. 3 Stimulation of *Hs* DNA polymerase γ by mtSSB. The stimulation assay was performed as described under Subheading 3, using 60 fmol of singly primed M13 DNA, 35 fmol of *Hs* Pol γA, 218 fmol of *Hs* Pol γB and increasing amounts of *Hs* mtSSB: 0, 6.4, 10.7, 16, and 32 pmol. The data were normalized to the nucleotide incorporated by *Hs* Pol γ in the absence of mtSSB

is a key component of the mitochondrial replisome, it is important to understand its properties during replication, and in particular, how it interacts with other key proteins of the replisome. To this end, we have developed an assay to evaluate the capacity of mtSSB to stimulate the DNA polymerase activity of Pol γ *in vitro* (Fig. 3) [5]. Similarly, we describe an assay to evaluate the stimulation of mtDNA helicase activity.

2 Materials

2.1 Human mtSSB Purification

1. pET11a encoding human mtSSB without the mitochondrial presequence (available from this lab).
2. *E. coli* BL21 (λDE3) pLysS.
3. Bacterial media (L broth).
4. 100 mg/mL Ampicillin (Filter sterilize and store at −20 °C).
5. 100 mM IPTG, stored at −20 °C.
6. 1 M Tris–HCl, pH 7.5, pH 8.0.
7. 2 M Tris–HCl, pH 6.8, pH 8.8.
8. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
9. 5 M Sodium chloride (NaCl).
10. 3 M Sodium thiocyanate (NaSCN).
11. 1 M Potassium phosphate (K_2HPO_4/KH_2PO_4), pH 7.6.
12. 80 % Sucrose, ultrapure.
13. 100 % Glycerol, anhydrous.
14. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Aliquots are stored at −20 °C.
15. 1 M Sodium metabisulfite, 1.0 M stock solutions at pH 7.5, stored at −20 °C.
16. 1 mg/mL Leupeptin prepared in 50 mM Tris–HCl, pH 7.5, 2 mM EDTA, stored at −20 °C.
17. 1 M Dithiothreitol (DTT), stored at −20 °C.
18. Sodium cholate.
19. 5 % Tween 20.
20. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4 °C.
21. 10 % Sodium dodecyl sulfate (SDS).
22. 4× Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS.
23. 4× Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
24. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
25. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at −20 °C.
26. 10 % Ammonium persulfate (APS).
27. *N,N,N′,N′*-tetramethylethylene-diamine (TEMED).
28. Blue Sepharose CL-6B (Amersham Pharmacia Biotech).

29. Phosphocellulose P-11 (Whatman) prepared as per manufacturer's directions.
30. Tris-sucrose buffer: 50 mM Tris–HCl, pH 7.5, 10 % sucrose.
31. 5× Lysis buffer: 1 M NaCl, 10 mM EDTA, 10 % sodium cholate.
32. Dilution buffer: 30 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA.
33. Blue Sepharose equilibration buffer: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.2 M NaCl.
34. Blue Sepharose wash buffer: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.25 M NaSCN.
35. Blue Sepharose elution buffer 1: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.4 M NaSCN.
36. Blue Sepharose elution buffer 2: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 1.2 M NaSCN.
37. Blue Sepharose elution buffer 3: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 1.5 M NaSCN.
38. Dialysis buffer: 60 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
39. Phosphocellulose equilibration buffer: 60 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
40. Phosphocellulose wash buffer: 60 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
41. Phosphocellulose elution buffer 1: 60 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
42. Phosphocellulose elution buffer 2: 150 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
43. Phosphocellulose elution buffer 3: 350 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA.
44. Centricon-30 spin concentrator.

2.2 GMSA of Human mtSSB

1. 48-mer oligonucleotide:
   5′-GGACTATTTATTAAATATATTTAAGAACTAATT CCAGCTGAGCGCCG G-3′.
2. T4 polynucleotide kinase.
3. 10× PNK buffer: 700 mM Tris–HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT.
4. [γ-^{32}P]ATP.
5. DE-81 Chromatography paper cut into 1 × 1 cm squares.
6. 0.3 M Ammonium formate, pH 7.5.
7. 95 % Ethanol.
8. Micro Bio-Spin P-30 Tris Chromatography column.

9. HumanmtSSB ~1 mg/mL in 35 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 20 % glycerol (*see* **Note 1**).
10. 1 M Tris–HCl, pH 7.5, pH 8.0.
11. 1 M Magnesium chloride ($MgCl_2$).
12. 5× SSB buffer: 100 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$.
13. 30 % Polyacrylamide (59:1; acrylamide:bisacrylamide), stored at 4 °C.
14. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
15. 5× TBE buffer: 450 mM Tris–HCl-borate, 10 mM EDTA.
16. 0.8 M Sodium chloride (NaCl).
17. 1 M Dithiothreitol (DTT).
18. 10× sample loading buffer: 50 % glycerol, 0.25 % bromophenol blue.
19. Phosphor Screen.
20. Storm 820 Scanner.

2.3 Assay of Stimulation of Pol γ Activity by HumanmtSSB

1. Pol γα 200 nM in 30 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 5 mM β-Mercaptoethanol, 20 % glycerol.
2. Pol γβ 275 nM in 30 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM β-Mercaptoethanol, 20 % glycerol.
3. mtSSB 20 μM in 35 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 20 % glycerol (*see* **Note 1**).
4. Singly-primed M13 DNA (6407 nt), 125 nM in 10 mM Tris–HCl, pH 8.0, 200 mM NaCl, 30 mM Na citrate, 8 mM EDTA.
5. Bovine serum albumin (BSA).
6. 1 M Tris–HCl, pH 8.5.
7. 1 M Magnesium chloride ($MgCl_2$).
8. 1 M Dithiothreitol (DTT).
9. 2 M Potassium chloride (KCl).
10. 100 % Glycerol, anhydrous.
11. Dilution buffer: 30 mM Tris–HCl, pH 7.5, 8 % glycerol.
12. 5× γN buffer: 250 mM Tris–HCl, pH 8.5, 20 mM $MgCl_2$, 2 mg/mL BSA.
13. 50× dNTPs mix: 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 0.5 mM dCTP.
14. α-^{32}P dCTP 10 μCi/μL.
15. 100 % Trichloroacetic acid (TCA).
16. 100 % Sodium pyrophosphate (NaPPi).
17. Stop solution (10 % TCA, 0,1 M NaPPi).
18. 12.1 M Hydrochloric acid (HCl).

19. Acid wash solution: 1 M HCl, 0,1 M NaPPi.
20. 95 % Ethanol.
21. Glass-fiber filter paper.
22. Glass vials, 20 mL.

3 Methods

3.1 Human mtSSB Purification

3.1.1 Bacterial Cell Growth and Protein Overproduction (See Note 2)

1. Use *E. coli* BL21 (λDE3) pLysS containing pET11a-human mtSSB (complete cDNA without the mitochondrial presequence) to inoculate 2 L of L broth containing 100 μg/mL ampicillin at $A_{595} = 0.06$, and grow with aeration at 37 °C.
2. After an $A_{595} = 0.6$ is reached:
   (a) Dispense a 1 mL aliquot of uninduced cells in a microcentrifuge tube. Centrifuge cells to pellet, remove supernatant and resuspend pellet in 200 μL of 1× SDS loading buffer. Use 10 μL of the aliquot as a control for the SDS-PAGE analysis.
   (b) Induce expression of target protein with the addition of IPTG to a final concentration of 0.2 mM and continue to incubate for 3 h at 37 °C.
3. Cell harvest:
   (a) Save a 1 mL aliquot of the induced cells as in **step 2a**. Use 5 μL for the SDS-PAGE analysis of protein overexpression. Use Coomassie Blue to stain the gel (*see* **Note 3**).
   (b) Harvest cells by spinning at 3600 × *g* for 5 min at 4 °C in Sorvall GSA bottles. Discard the supernatant.
   (c) Resuspend resulting pellets in 1/10 volume of the original culture with Tris-sucrose buffer. Use one-half of the total resuspension volume to resuspend pellets, then transfer to a pre-chilled beaker. With the remaining one-half of the volume wash the GSA bottles and combine in the beaker.
   (d) Divide the washed cells into Sorvall SS-34 tubes, and centrifuge at 3000 × *g* for 5 min at 4 °C. Remove the supernatant and freeze the resulting pellets in liquid nitrogen. Store at −80 °C.

3.1.2 Cell Lysis and Preparation of Soluble Fraction

1. Thaw cell pellets on ice for ≥30 min.
2. Resuspend cell pellet in 1/25 volume of Tris-sucrose buffer as described above.
3. Add 5× lysis buffer to a 1× final concentration and incubate on ice for 30 min with gentle inversions every 5 min.

4. Use liquid nitrogen to freeze cell suspension, thaw partially in ice water, then transfer to ice until completely thawed.
5. Centrifuge cells at 17,500 × *g* for 30 min at 4 °C in Sorvall SS-34 tubes.
6. Collect supernatant (soluble fraction, Fraction I) by pipetting into a fresh, pre-chilled tube.
7. Use dilution buffer to adjust the sample to 200 mM NaCl equivalent.

3.1.3 Blue Sepharose Chromatography

1. Pack a Blue Sepharose column (5–7 mg of total protein/mL of resin) and equilibrate with 10 column volumes (CV) of equilibration buffer at a flow rate of 1 CV/h.
2. Load the salt-adjusted sample onto the column at a flow rate of 1 CV/h.
3. Wash column with 1 CV of wash buffer at a flow rate of 2 CV/h, collecting 1 CV fractions.
4. Elute column with 8 CV of the elution buffers containing 0.4–1.2 M NaSCN in a linear gradient at 2 CV/h. Collect 1/6 CV fractions.
5. Perform a final elution step with 2 CV of elution buffer containing 1.5 M NaSCN. Collect 1/4 CV fractions (*see* **Note 4**).
6. Analyze fractions by SDS-PAGE on 17 % minigels followed by Coomassie Blue staining, and pool fractions accordingly (Fraction II).
7. Dialyze Fraction II against buffer containing 60 mM KPO_4, pH 7.6, 10 % glycerol, 2 mM EDTA, until the fraction is at an ionic equivalent of 60 mM KPO_4 (Fraction IIb).

3.1.4 Phosphocellulose Chromatography (See Note 1*)*

1. Pack a phosphocellulose column (0.5 mg of total protein/mL of resin) and equilibrate with 10 column volumes (CV) of equilibration buffer at a flow rate of 1 CV/h.
2. Load Fraction IIb onto the column at a flow rate of 0.8 CV/h.
3. Wash column with 2.5 CV of wash buffer at a flow rate of 2 CV/h, collecting 1/2 CV fractions.
4. Elute column with 5 CV of the elution buffers containing 60–150 mM KPO_4 in a linear gradient at 2 CV/h. Collect 1/6 CV fractions.
5. Perform a final elution step with 2 CV of elution buffer containing 350 mM KPO_4. Collect 1/4 CV fractions (*see* **Note 5**).
6. Analyze fractions by SDS-PAGE on 17 % minigels followed by Coomassie Blue staining, and pool fractions accordingly (Fraction III).

7. Use a Centricon 30 concentrator (pre-treated with 5 % Tween 20 overnight at room temperature) to spin concentrate the sample to ~1 mg/mL, (Fraction IIIb) (*see* **Note 6**).
8. Aliquot Fraction IIIb into microcentrifuge tubes, freeze in liquid nitrogen and store at –80 °C.

3.2 Electrophoretic Mobility Shift Assay of HumanmtSSB

1. The 50 μL kinase reaction contains 1× PNK buffer, [γ-^{32}P]ATP (0.44 μM, 4500 Ci/mmol), 340 pmol (as nt) of oligonucleotide, and 20 units of T4 polynucleotide kinase.
2. Incubate the reaction for 30 min at 37 °C.

3.2.1 5′-End Labeling of 48-mer Oligodeoxyribonucleotide

3. Spot 0.25 μL of the reaction onto 5 squares of DE-81chromatography paper (1 × 1 cm). Wash 3 of the squares in 0.3 M ammonium formate, twice for 5 min, then once in 95 % ethanol. Dry all 5 squares under a heat lamp for 5 min. Count radioactivity on each square in scintillation fluid, and calculate incorporation (*see* **Note 7**).
4. Purify labeled oligonucleotide using a Micro Bio-Spin P-30 Tris chromatography column.

3.2.2 Gel Mobility Shift Assay

1. Prepare a master mix such that each reaction contains 1× SSB buffer, 1 mM DTT, 36 fmol of ^{32}P-48-mer substrate, and 50 mM NaCl. Prepare the reaction mixture in a microcentrifuge tube, heat the mix to 100 °C for 5 min, vortex and centrifuge briefly.
2. Dispense the mix into microcentrifuge tube(s) on ice. Adjust with water for a final volume of 20 μL after considering the addition of desired amount of the mtSSB.
3. Add the desired amount of mtSSB (for example: 0, 1, 2, 4, 6, 10, and 15 nM as tetramer) to the microcentrifuge tube(s) and incubate at 20 °C for 10 min.
4. Add 2 μL of 10× sample buffer to the reaction(s).
5. Load sample(s) onto a pre-run (200 V for 2 h) 6 % native polyacrylamide gel and electrophorese for 3 h at 200 V in 1× TBE (*see* **Note 8**).
6. Dry gel under vacuum with heat, and expose to a Phosphor Screen from 4 h to overnight.
7. Scan Phosphor Screen using the Storm 820 scanner. Determine the volume of each band and subtract background using computer integration analysis (*see* **Note 9**).

3.3 Assay of Stimulation of Pol γ Activity by HumanmtSSB

1. Adjust a water bath to 37 °C.
2. Prepare a master reaction mix such that each 25 μL reaction contains 1× γN buffer, 10 mM DTT, 1× dNTPs, 2.4 nM M13 template, 2 μCi α-^{32}P dCTP, and 15 mM KCl.

3. Dispense the mix into pre-chilled microcentrifuge tube(s) on ice. Adjust with water for a final volume of 25 μL after considering the addition of desired amounts of the mtSSB and Pol γ.
4. Add the desired amount of mtSSB as 5 μL aliquots (for example, the final concentration of: 0.25, 0.40, and 0.65 μM as tetramer) to the microcentrifuge tubes. Include a reaction with no mtSSB as a control.
5. Prepare dilutions of Pol γα and β at 7.2 and 40 nM, respectively. Mix equal volumes of the protein dilutions and dispense 10 μL into the tubes containing the master mix and mtSSB (*see* **Note 10**).
6. Incubate the tubes for 30 min at 37 °C, then transfer to ice.
7. Stop the reactions with 0.5 mL of stop solution and incubate on ice for 5 min.
8. Filter samples through glass-fiber filters. Wash the reaction tube twice with acid wash solution, then wash the filtration funnel three times with acid wash solution and once with 95 % ethanol.
9. Dry the filters under a heat lamp for 5 min, then count in scintillation fluid.
10. Spot 1 μL of mix directly onto two glass fiber filters, dry, and count in scintillation fluid without filtration to calculate the specific radioactivity of the mix and determine nucleotide incorporation (*see* **Note 11**).

4 Notes

1. Following the Blue Sepharose chromatography step, if the presence of a dsDNA-unwinding contaminant is not a concern (e.g., as is the case for GMSA and the assay for stimulation of DNA polymerase activity), the protein can be purified further using glycerol gradient sedimentation [9].
2. Using the pET11 system, we express 50–100 mg of mtSSB protein per liter of cell culture.
3. The SDS-PAGE gel must be stained with Coomassie Blue. A silver-stained gel is not suited for detection of human mtSSB, especially when analyzing the level of induction in the bacterial cell extract.
4. The wild-type *Hs*mtSSB can be eluted from the Blue Sepharose column with NaCl by increasing the ionic strength of the elution buffers [9].
5. *Hs*mtSSB proteins elute at ~80 mM KPO_4.

6. After overnight treatment with 5 % Tween 20, rinse thoroughly with Milli-Q water.
7. Incorporation is calculated as: [(average cpm of three washed papers)/(average cpm/pmol of unwashed papers)]/pmol ends in the reaction.
8. Use a fan for cooling, dye-front migration should be approximately 10 cm.
9. The amounts of shifted and free oligonucleotide are calculated as: % ssDNA bound = $[V_S/(V_S + V_F)] \times 100$; V_S represents the volume of the shifted and V_F represents the volume of unshifted oligonucleotide in the lane of interest.
10. We use an ~sixfold molar excess of the Pol γβ dimer over Pol γα to ensure complete reconstitution of Pol γ.
11. The DNA polymerase activity of Pol γ is measured as pmol of nucleotides incorporated into the nascent DNA strand. The molar amount of incorporated nucleotide is calculated as follows:
    (a) Incorporated nucleotide = average cpm of washed filters/[(average cpm/μL of unwashed filters)/(pmol/μL of nucleotides spotted)]
    (b) To obtain fold of stimulation, calculate the ratio of nucleotide incorporation in the samples containing mtSSB as compared to those without mtSSB.

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Chapter 17

Biolayer Interferometry: A Novel Method to Elucidate Protein–Protein and Protein–DNA Interactions in the Mitochondrial DNA Replisome

Grzegorz L. Ciesielski, Vesa P. Hytönen, and Laurie S. Kaguni

Abstract

A lack of effective treatment for mitochondrial diseases prompts scientists to investigate the molecular processes that underlie their development. The major cause of mitochondrial diseases is dysfunction of the sole mitochondrial DNA polymerase, DNA polymerase γ (Pol γ). The development of treatment strategies will require a detailed characterization of the molecular properties of Pol γ. A novel technique, biolayer interferometry, allows one to monitor molecular interactions in real time, thus providing an insight into the kinetics of the process. Here, we present an application of the biolayer interferometry technique to characterize the fundamental reactions that Pol γ undergoes during the initiation phase of mitochondrial DNA replication: holoenzyme formation and binding to the primer-template.

Key words Biolayer interferometry, Protein–protein interactions, Mitochondria, Mitochondrial replisome

1 Introduction

Mitochondria are essential organelles that play a key role in fundamental cellular processes, such as oxidative phosphorylation (OXPHOS), calcium signaling, and apoptosis. Human mitochondria cotntain a semi-independent genome that encodes only 13 polypeptides, all of which are crucial components of the OXPHOS machinery [1]. Thus, defects in the mitochondrial genome often result in a shortage of ATP, which affects tissues with high energy requirements (such as the nervous system or muscle tissue) and can lead to the development of so-called mitochondrial diseases [2]. There is no known cure for any of the diseases resulting from mtDNA dysfunction and only symptom treatment strategies are currently available [3–7]. The majority of human mitochondrial disorders originate from dysfunction of the sole mitochondrial DNA polymerase, DNA polymerase γ (Pol γ). To date, nearly 250

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disease-causing mutations have been identified within the *POLG* gene, encoding the catalytic subunit of Pol γ, Pol γα (http://tools.niehs.nih.gov/polg/). On the molecular level, Pol γ dysfunction causes mtDNA depletion and multiple mtDNA deletions. The mechanisms of pathogenesis of the POLG syndromes are largely unknown and can result from various Pol γ-related issues, such as defects in assembly of the Pol γ holoenzyme, problems in recognition of the primer-template, decreased processivity, etc. Each of these functions is based on specific molecular interactions between all the factors contributing to Pol γ function. Understanding these interactions is thus fundamental to elucidate the molecular basis of disease, and to develop treatments [1].

Pol γα is an ~140 kDa subunit that catalyzes the nucleotide polymerization reaction. It has a weak intrinsic single-stranded DNA (ssDNA) binding ability, but it is able to recognize and bind primer-template and synthesize ~200 nt of nascent strand per turnover [8]. It has also been suggested that Pol γα plays an independent role in mtDNA repair processes [9]. In the replication process, Pol γα binds to its homodimeric accessory subunit, Pol $\gamma\beta_2$ (55 kDa/promoter), to form the Pol γ holoenzyme [10]. Pol γβ subunit enhances the ssDNA-binding affinity of Pol γα by approximately 100-fold, and increases its processivity by 50–100-fold [11, 12]. Pol γβ also has the ability to bind dsDNA on its own [13], though this appears to be dispensable for its function as a component of Pol γ; as a part of the holoenzyme, the Pol γβ dimer does not contact the DNA template directly [14]. The low abundance of both of these proteins *in vivo* limits studies of the early steps of mtDNA replication. Thus, little is known about the mechanisms leading to initiation of the replication process.

In this chapter, we describe the use of biolayer interferometry to study two crucial stages of the initiation process: the formation of Pol γ holoenzyme and replication site recognition. Biolayer interferometry is a technique based on the optical phenomenon of wave interference. It utilizes a novel type of biosensor in the form of a tip with two specific layers at its end. The first external layer, called the biolayer, is coated with molecules of interest and the second layer is an internal reference optical layer. During the measurement, white light is streamed constantly along the biosensor fiber and is reflected from the reference optical layer, as well as from the biolayer. Superposition of waves reflected from both layers forms the interference pattern. Binding of a ligand to molecules on the biolayer changes its optical properties and thus alternates the phase of the reflected light. This effect causes a wavelength shift in the interference pattern, which correlates directly with the thickness of the biosensor tip. The interference pattern is measured constantly, allowing one to monitor changes of the biosensor thickness (nm) in real time [15, 16]. In practice, executing an experiment is limited to the preparation of solutions with and

without ligands of interest, in a 96- or 384-well black plate. The Octet device places biosensors into solutions as programmed and displays the results of the analysis in real time, enabling the determination of binding kinetics and affinity. The biosensors are available with diverse modifications of the biolayer, e.g., streptavidin, Ni-NTA, anti-GST antibody, protein A, etc. (http://www.fortebio.com/). This relatively novel technique has already been applied successfully in studies of protein–protein interactions, such as interactions between protein components of the iron-sulfur cluster assembly pathway, or protein–DNA interactions, such as binding of the XPD helicase to ssDNA [17, 18].

Interaction of the Pol γα and Pol $\gamma\beta_2$ subunits is fundamental to the formation of Pol γ holoenzyme, and has been studied in the past [11, 19]. To evaluate biolayer interferometry for studies of the mtDNA replisome, we load His-tagged Pol γα onto Ni-NTA-coated biosensors, move the sensors to a reaction buffer containing bovine serum albumin (BSA), which limits non-specific interactions with any unbound sites. BSA is also thought to inhibit weak protein–protein interactions, and is thus considered useful to block non-specific binding between proteins. The response reached with the blocking step serves as a baseline for subsequent steps. Next, sensors are placed into solutions of various Pol γβ concentrations to evaluate the association step, and subsequently placed back into the blocking/baseline solution for the determination of dissociation properties (Fig. 1). The data collected are processed and analyzed with the Octet Data Analysis Software.

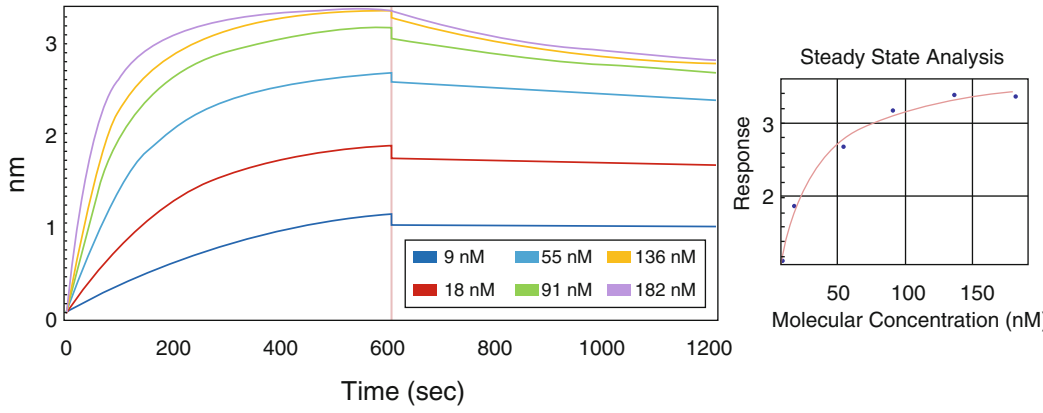

Fig. 1 Biolayer interferometry analysis of the Pol γ α–β interaction. Ni-NTA sensors were loaded with Pol γα-his_6 exo^- (10 μg/mL) and blocked with BSA. During the association step (from 0 to 600 s), the sensors were placed into Pol γβ solutions of various concentrations: 9, 18, 55, 91, 136, and 182 nM. Subsequently, the sensors were moved to the buffer without Pol γα or β for dissociation analysis (from 600 to 1200 s). The data were processed and analyzed with the Octet Data Analysis Software. A control without Pol γβ was subtracted from remainder of the data collected. The *right panel* represents steady-state analysis of the response (nm) to Pol γβ concentrations (nM)

Earlier studies of Pol γ holoenzyme formation reported that the subunits interact with apparent dissociation constant (K_D) of 35 nM (±16 nM) [19]. This value was estimated as a function of Pol γβ concentration and its measured biochemical activity of stimulating maximal DNA binding and nucleotide polymerization by Pol γα. For comparison, we utilized a similar approach and used a steady-state analysis for K_D estimation, measuring the association response that yielded a value of 20 nM (±1.5 nM). The interaction of the subunits was also analyzed with surface plasmon resonance (SPR) in an earlier study [11]. On a basis of kinetic analysis of association and dissociation rate constants, the authors estimated the K_D value to be 27 nM, which is similar to the value we obtain with the steady-state analysis by biolayer interferometry. Because the real-time measurements by biolayer interferometry enable kinetic analysis as well, we fit our data to 1:1 model and obtained association ($k_{on} = 1.03 \times 10^5\ 1/M \times s$) and dissociation rates ($k_{off} = 3.02 \times 10^{-4}\ 1/s$), with a resulting K_D value of 2.95 nM (±0.03 nM). Although the binding affinity determined by the two different fitting methods vary substantially, they both indicate a tight interaction between the subunits in good agreement with earlier studies. Notably, the data presented here suggest that once assembled, the Pol γ subunits dissociate at a very limited rate. The stability of the Pol γ holoenzyme is further supported by previous studies utilizing crystallography and other biophysical techniques [10, 20]. In comparison to the previous SPR studies [11], in the experiments presented here Pol γβ associates faster with Pol γα to reach the equilibrium state, and dissociates significantly slower. The main difference between our approach and those in the previous studies lies in the preparation and regeneration of the Pol γα-coated sensors (*see* **Note 1**). In the SPR experiments the authors used an amino-coupling approach and regeneration with 3 mM NaOH, which might impact the native conformation of the enzyme. The use of Ni-NTA sensors allow us to prepare Pol γα-coated sensors by a gentle immobilization strategy, and the low cost of sensors makes it possible to eliminate a regeneration step.

The Octet biosensor also makes it possible to study protein–DNA interactions. To study the interaction of Pol γ with a primer-template DNA, we used a 5′-biotin-labeled 40-mer oligonucleotide annealed to an 80-mer oligonucleotide, forming a template of 40 nt dsDNA with a free 3′-OH end followed by 40 nts stretch of ssDNA. Streptavidin biosensors were loaded with the primer-template DNA and blocked with BSA. The affinity of the Pol γ holoenzyme to the primer-template DNA was measured by placing the sensors into solution of various Pol γ holoenzyme concentrations. After the association step the sensors are placed back to the baseline solution for the determination of dissociation (Fig. 2), and the data collected are processed and analyzed with the Octet Data Analysis Software.

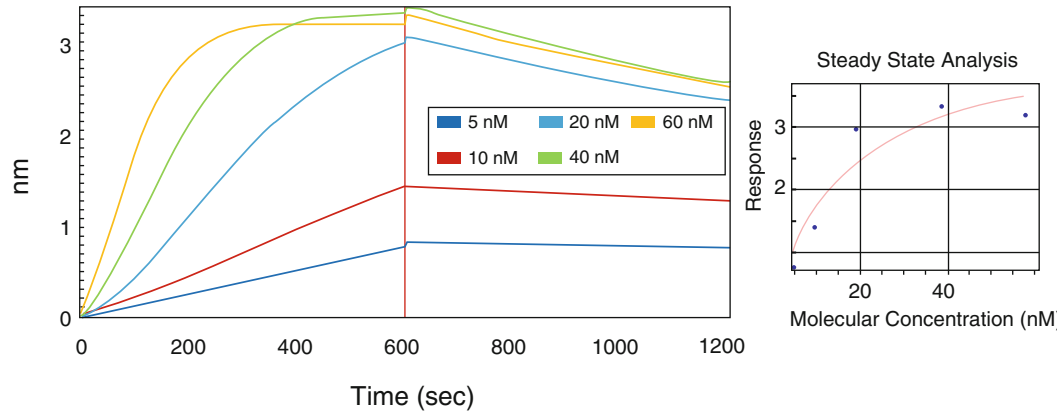

Fig. 2 Biolayer interferometry analysis of the Pol γ–primer-template DNA interaction. Streptavidin biosensors were presoaked and loaded with 25 nM 5′-biotinylated 40/80-mer primer-template DNA. After blocking, sensors were placed into Pol γ solutions of 5, 10, 20, 40, and 60 nM, prepared as a mixture of α to β_2 subunits in a 1–1.5 molar ratio (association—0–600 s). The dissociation rate was determined after the association step by placing the sensors back into the buffer without Pol γα or β (600–1200 s). The data obtained were processed and analyzed with the Octet Data Analysis Software. A control without Pol γ subunits was subtracted from the remaining set of raw data. The *right panel* represents steady-state analysis of the response (nm) to Pol γβ concentrations (nM)

Previously published K_D values for the Pol γ–primer-template DNA exo– interaction vary significantly depending on the method and conditions used (*see* **Note 2**). Kinetic analysis using SPR yielded a value of 0.06 nM, whereas results of gel mobility shift assay and analysis of the stimulation of DNA binding of Pol γα by Pol γβ yielded a value of ~10 nM [11, 12, 19]. The results we obtained with biolayer interferometry analyzed as a function of concentration and association response yielded a K_D of 16 nM (±6.5 nM). In the kinetic analysis we determined association and dissociation rates ($k_{on} = 1.06 \times 10^5$ 1/M×s, $k_{off} = 3.99 \times 10^{-4}$ 1/s), fitting the results to 1:1 binding model, and obtained a K_D value of 3.76 nM (±0.06 nM). Dissociation constant values obtained with both approaches are thus similar to those published previously, except for those determined by SPR. The difference obtained could be linked to the immobilization strategy. In our approach, streptavidin sensor places the DNA strands sparsely and the layer thickness is low (according to manufacturer the biosensor surface may be considered almost flat). We also noticed that the Pol γ–DNA binding data show some deviation from a 1:1 binding model, which could indicate more a complex binding mechanism. Therefore, further studies are needed to understand all the details of the interaction.

In summary, these examples demonstrate that biolayer interferometry provides an effective tool for studies of the mitochondrial replisome. Future studies could be developed to examine

how mutations in either of the Pol γ subunits affect holoenzyme formation or the primer-template binding properties of the enzyme, or to test small molecules that may stimulate or inhibit mtDNA replication.

2 Materials

The biolayer interferometry analyses were performed with the ForteBio Octet RED 384 system. The data collected were processed and analyzed using the Octet Data Analysis Software.

2.1 Pol γ Holoenzyme Formation

1. Pol γα-his_6 exo–, 0.04 μg/μL (*see* **Note 1**).
2. Pol γβ, 0.05 μg/μL.
3. Bovine serum albumin (BSA).
4. 1 M Tris–HCl, pH 7.5.
5. 2 M Potassium chloride (KCl).
6. 1 M Magnesium chloride ($MgCl_2$).
7. Tween 20.
8. Glycerol.
9. Ni-NTA (NTA) biosensors.
10. 384-Well, tilted-bottom black plate (*see* **Note 3**).
11. Pol γα buffer: 30 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 20 % glycerol, 5 mM β-Mercaptoethanol.
12. Reaction buffer 1: 50 mM Tris–HCl, pH 7.5, 1 μg/μL BSA, 4 mM $MgCl_2$, 0.02 % Tween 20 (*see* **Note 1**).

2.2 Binding of Pol γ to Primer-Template DNA

1. Pol γα-his_6, 0.08 μg/μL (*see* **Note 2**).
2. Pol γβ, 0.05 μg/μL.
3. 1 μM solution of 5′-biotin-labeled 40 bases oligonucleotide:
   5′-ATT ATG AAT TAA TTT AAT AAT TTT TTT TTT TTT TTT TTT T-3′.
4. 1.2 μM solution of 80-mer oligonucleotide:
   5′-GGG GGG GGG GGG GGG GGG GGC TGA CTG GGC GGC TCA CTG GAA AAA AAA AAA AAA AAA AAA TTA TTA AAT TAA TTC ATA AT-3′.
5. Streptavidin (SA) biosensors.
6. 384-Well, tilted-bottom black plate (*see* **Note 3**).
7. Phosphate-buffered saline (PBS): 135 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4.

8. Pol γα buffer: 30 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 20 % glycerol, 5 mM β-Mercaptoethanol.
9. Reaction Buffer 2: 50 mM Tris–HCl, pH 8.0, 0.4 μg/μL BSA, 10 mM DTT, 50 mM KCl 4 mM $MgCl_2$ (*see* **Note 2**).

3 Methods

3.1 Pol γ Holoenzyme Formation

1. Place NTA sensors in a tray and soak them in Pol γα buffer for 30 min.
2. Prepare a master solution of 10 μg/mL Pol γα-his_6 exo in Pol γα buffer (*see* **Note 3**).
3. Prepare Pol γβ solutions of 9, 18, 55, 91, 136, and 182 nM in reaction buffer 1. Also include a control without Pol γβ.
4. Pipette solutions for each step in individual wells of a 384-well, tilted-bottom black plate. Measure the following steps:
   Baseline—40 μL Pol γα buffer.
   Loading—40 μL Pol γα solution.
   Baseline 2 and dissociation—40 μL reaction buffer 1.
   Association—40 μL Pol γβ solutions of various concentrations.
5. Set the kinetic experiment parameters to 1000 rpm for each step and set duration of steps:
   Baseline—60 s.
   Loading—300 s.
   Baseline 2–300 s.
   Association—600 s.
   Dissociation—600 s.
6. Start the measurement.
7. Process the data as follows:
   Set data from no-Pol γβ control well as reference, and subtract it from the remaining wells.
   Align Υ axis to the last 5 s of baseline 2.
   Set the inter-step correlation to dissociation.
   Apply the Savitzky–Golay transformation.
8. Analyze the data by applying the following settings:
   Set both association and dissociation to be analyzed.
   1:1 Binding model.
   Apply global fitting with Rmax Unlinked.

3.2 Binding of Pol γ to Primer-Template DNA

1. Prepare template master solution by annealing 40-mer and 80-mer oligos. Mix equal volumes of the oligomer solutions and incubate for 30 min at 90 °C. After incubation allow the sample to cool down to room temperature.
2. Prepare 25 nM primer-template solution in PBS (1 pmol/40 μL).

3. Place SA sensors in a tray and soak them in PBS for 30 min.
4. Prepare 60 nM Pol γ solution mixing Pol γα and Pol $\gamma\beta_2$ solutions in a 1–1.5 molar ratio, respectively, and then prepare dilutions in reaction buffer 2 of 5, 10, 20, and 40 nM. Include a control without Pol γ.
5. Pipette solutions for each step into individual wells of a 384-well, tilted-bottom black plate:
   Baseline—40 μL PBS.
   Loading—40 μL of primer-template solution.
   Baseline 2 and dissociation—40 μL reaction buffer 2.
   Association—40 μL of individual Pol γ solutions.
6. Set the kinetic experiment parameters by setting the shaking rate at 1000 rpm and the duration of each step as follows:
   Baseline—60 s.
   Loading—300 s.
   Baseline 2–300 s.
   Association—600 s.
   Dissociation—600 s.
7. Start the measurement.
8. Process and analyze the data as in the previous section using the no-Pol γ control as a reference.

4 Notes

1. The Pol γ holoenzyme formation experiment was performed under conditions similar to those used previously for SPR studies [11].
2. The Pol γ–primer-template DNA interaction experiment was performed under conditions similar those used for the Pol γ activity assay [12].
3. The 96-well black plate could be used as well, but in that case every solution should be scaled up to 200 μL. The 384-tilted well plate may improve the quality of the baseline and reduce the deviation in the baseline level between the wells.

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ERRATUM TO

Quantitation of Mitochondrial DNA Deletions Via Restriction Digestion/Long-Range Single-Molecule PCR

Yevgenya Kraytsberg, Xinhong Guo, Saisai Tao, Alexandra Kuznetsov, Catherine MacLean, Daniel Ehrlich, Evan Feldman, Igor Dombrovsky, Deye Yang, Gregory J. Cloutier, Carmen Castaneda-Sceppa, and Konstantin Khrapko

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The last name of the author of Chapter 4 was misspelled as "McLean" while the correct spelling is "MacLean"

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