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.

Matthew McKenzie (ed.), *Mitochondrial DNA: Methods and Protocols*, Methods in Molecular Biology, vol. 1351,
DOI 10.1007/978-1-4939-3040-1_13,

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.

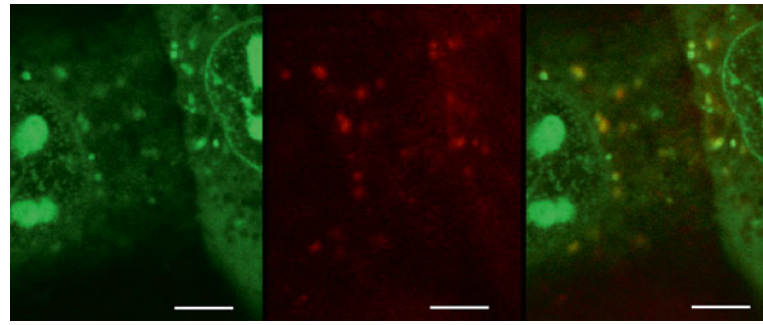

Fig. 1 Fluorescence *in vivo* hybridization of HepG2 cells stained with Syto 9 dye (*green*) to visualize mitochondrial nucleoids and nuclei. Cells were transfected as described in this protocol with probe containing MAM sequence, hybridization sequence complementary to mtND5 gene and AlexaFluor® 647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

Acknowledgement

This work was supported by the Czech Science Foundation grant No. P305/12/P388 to JZ and by the research projects RVO67985823 and BIOCEV—Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0109), the European Regional Development Fund.

References

1. Li H, Liu D, Lu J, Bai Y (2012) Physiology and pathophysiology of mitochondrial DNA. Adv Exp Med Biol 942:39–51
2. Kasiviswanathan R, Collins TR, Copeland WC (2012) The interface of transcription and DNA replication in the mitochondria. Biochim Biophys Acta 1819:970–978
3. Holt IJ (2009) Mitochondrial DNA replication and repair: all a flap. Trends Biochem Sci 34:358–365
4. Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. Annu Rev Biochem 76:679–699
5. Niazi AK, Mileshina D, Cosset A, Val R, Weber-Lotfi F, Dietrich A (2013) Targeting nucleic acids into mitochondria: progress and prospects. Mitochondrion 13:548–558
6. Durazo SA, Kompella UB (2012) Functionalized nanosystems for targeted mitochondrial delivery. Mitochondrion 12:190–201
7. Comte C, Tonin Y, Heckel-Mager AM, Boucheham A, Smirnov A, Auré K et al (2013) Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. Nucleic Acids Res 41:418–433
8. Jash S, Adhya S (2012) Induction of muscle regeneration by RNA-mediated mitochondrial restoration. FASEB J 26:4187–4197
9. Sieber F, Duchene AM, Marechal-Drouard L (2011) Mitochondrial RNA import: from diversity of natural mechanisms to potential applications. Int Rev Cell Mol Biol 287:145–190
10. Weber-Lotfi F, Ibrahim N, Boesch P, Cosset A, Konstantinov Y, Lightowlers N, Dietrich RNA (2009) Developing a genetic approach to investigate the mechanism of mitochondrial competence for DNA import. Biochim Biophys Acta 1787:320–327
11. Mahata B, Mukherjee S, Mishra S, Bandyopadhyay A, Adhya S (2006) Functional delivery of a cytosolic tRNA into mutant mitochondria of human cells. Science 314:471–474
12. D'Souza GG, Rammohan R, Cheng SM, Torchilin VP, Weissig V (2003) DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells. J Control Release 92:189–197
13. Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y et al (2008) MITO-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim Biophys Acta 1778:423–432
14. Muratovska A, Lightowlers RN, Taylor RW, Turnbull DM, Smith RA, Wilce JA et al (2001) Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expression and disease. Nucleic Acids Res 29:1852–1863
15. Magalhaes PJ, Andreu AL, Schon EA (1998) Evidence for the presence of 5S rRNA in mammalian mitochondria. Mol Biol Cell 9:2375–2382
16. Rorbach J, Minczuk M (2012) The post-transcriptional life of mammalian mitochondrial RNA. Biochem J 444:357–373
17. Smirnov A, Entelis N, Martin RP, Tarassov I (2011) Biological significance of 5S rRNA import into human mitochondria: role of ribosomal protein MRP-L18. Genes Dev 25:1289–1305
18. Smirnov AV, Entelis NS, Krasheninnikov IA, Martin R, Tarassov IA (2008) Specific features of 5S rRNA structure – its interactions with macromolecules and possible functions. Biochemistry (Mosc) 73:1418–1437
19. Smirnov A, Tarassov I, Mager-Heckel AM, Letzelter M, Martin RP, Krasheninnikov IA,

Entelis N (2008) Two distinct structural elements of 5S rRNA are needed for its import into human mitochondria. RNA 14:749–759

20. Tarassov I, Kamenski P, Kolesnikova O, Karicheva O, Martin RP, Krasheninnikov IA, Entelis N (2007) Import of nuclear DNA-encoded RNAs into mitochondria and mitochondrial translation. Cell Cycle 6:2473–2477
21. Smirnov A, Comte C, Mager-Heckel AM, Addis V, Krasheninnikov IA, Martin RP et al (2010) Mitochondrial enzyme rhodanese is essential for 5S ribosomal RNA import into human mitochondria. J Biol Chem 285:30792–30803
22. Vedrenne V, Gowher A, De Lonlay P, Nitschke P, Serre V, Boddaert N et al (2012) Mutation in PNPT1, which encodes a polyribonucleotide nucleotidyltransferase, impairs RNA import into mitochondria and causes respiratory-chain deficiency. Am J Hum Genet 91:912–918
23. Wang G, Shimada E, Koehler CM, Teitell MA (2012) PNPASE and RNA trafficking into mitochondria. Biochim Biophys Acta 1819:998–1007
24. Wang G, Chen HW, Oktay Y, Zhang J, Allen EL, Smith GM et al (2010) PNPASE regulates RNA import into mitochondria. Cell 142:456–467
25. Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA et al (2012) Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A 109:4840–4845
26. Sieber F, Placido A, El Farouk-Ameqrane S, Duchene AM, Marechal-Drouard L (2011) A protein shuttle system to target RNA into mitochondria. Nucleic Acids Res 39:e96
27. Zelenka J, Alán L, Jabůrek M, Ježek P (2014) Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent in vivo hybridization of mitochondrial DNA and RNA. J Bioenerg Biomembr 46:147–156
28. Wu TP, Ruan KC, Liu WY (1996) A fluorescence-labeling method for sequencing small RNA on polyacrylamide gel. Nucleic Acids Res 24:3472–3473