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Yeast Mitochondrial Transcriptomics

Mathilde Garcia, Xavier Darzacq, Frederic Devaux, Robert H. Singer, and Claude Jacq

Summary

Although 30 years ago it was strongly suggested that some cytoplasmic ribosomes are bound to the surface of yeast mitochondria, the mechanisms and the raison d'être of this process are not understood. For instance, it is not perfectly known which of the several hundred nuclearly encoded genes have to be translated to the mitochondrial vicinity to guide the import of the corresponding proteins. One can take advantage of several modern methods to address a number of aspects of the site-specific translation process of messenger ribonucleic acid (mRNA) coding for proteins imported into mitochondria. Three complementary approaches are presented to analyze the spatial distribution of mRNAs coding for proteins imported into mitochondria. Starting from biochemical purifications of mitochondria-bound polysomes, we describe a genomewide approach to classify all the cellular mRNAs according to their physical proximity with mitochondria; we also present real-time quantitative reverse transcription polymerase chain reaction monitoring of mRNA distribution to provide a quantified description of this localization. Finally, a fluorescence microscopy approach on a single living cell is described to visualize the in vivo localization of mRNAs involved in mitochondria biogenesis.

Key Words: DNA microarrays; mRNA localization; Q-RT-PCR; single-cell FISH.

1. Introduction

The complex cellular processes that supervise the building of mitochondria are not all identified. The protein import process is probably the best-clarified step. Three decades of smart biochemical experiments, mainly conducted in yeast, have led to a precise description of the machineries involved ***(1,2)***. One of the next objectives will be to integrate these biochemical concepts into a living process in which each step will be regulated according to cellular requirements. In that respect, the question of the process by which cytoplasmically translated proteins are delivered to mitochondria is still a matter of speculation. It was

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suggested ***(3)*** that proteins could be translated to the vicinity of mitochondria, and that cotranslational protein import into mitochondria could represent an alternative view ***(4)*** to an uncoupled process. One the other hand, the well-established evidence that some mitochondrial proteins can be imported in vitro has motivated the general belief that mitochondria-bound polysomes may not represent an obligatory process and, to some extent could be experimental artifacts ***(5)***.

However, several experiments conducted with new methodologies have considerably revitalized this question. First, by means of a genomewide approach, it was shown ***(6)*** that a large proportion of messenger ribonucleic acids (mRNAs) for nuclear-encoded, mitochondrially localized proteins are translated to the vicinity of mitochondria. The fact that a strong correlation was found ***(6)*** between the genes with mRNA translated to the vicinity of mitochondria and their prokaryotic origin (as suggested in **ref. *7***) gives credence to the results of microarray analyses. After these analyses, a mitochondrial localization of RNA (MLR) value ranging from 0 to 100 was given. Genes with a high MLR value (80–100) correspond to mRNAs likely to be found to the vicinity of mitochondria. In vivo fluorescent microscopy analyses have established that this corresponds to the actual cellular spatial distribution of these mRNAs ***(6)***. Finally, genetic experiments conducted with the gene *ATP2* have shown that this specific mRNA spatial distribution is highly dependent on the integrity of its 3′-UTR sequence, which is also required for correct and functional mitochondrial activity ***(6)***.

The putative role of restricted translation process in the biogenesis of mitochondria can be addressed through different strategies. We present three powerful experimental approaches that should shed new complementary light on this interesting question.

2. Materials

The strain CW252 ***(8)*** isogenic to W303 should be favored because of its intron-less mitochondrial genome, allowing easier detection of mitochondrial transcripts.

2.1. Mitochondria-Bound and Total RNA Isolation for Quantitative Polymerase Chain Reaction and Microarrays

1. YPGal: 1% (w/v) Bacto™ peptone, 1% (w/v) Bacto yeast extract, 2% (w/v) galactose. Autoclave 30 min at 110°C. Store at room temperature.
2. Gal-rich medium: 1% (w/v) Bacto peptone, 1% (w/v) Bacto yeast extract, 2% (w/v) galactose, 0.1% (w/v) KH_2PO_4, 0.12% (w/v) $(NH_4)_2SO_4$. 1 mL mix is made and dispensed in two 2-mL Erlenmeyer flasks and then autoclaved for 30 min at 110°C.
3. Preincubation buffer: 100 m*M* Tris-HCl, pH 9.3, 0.5 *M* β-mercaptoethanol. Do not store; should be prepared just before use.

4. Digestion buffer: 20 m*M* potassium phosphate buffer, pH 7.4, 1.35 *M* sorbitol. Do not store; should be prepared the day before the mitochondria isolation.
5. Zymolyase 100T (Seikagatu Corp., 120493).
6. Washing medium: 1% (w/v) Bacto peptone, 1% (w/v) Bacto yeast extract, 1 *M* sorbitol, 0.1% (w/v) KH_2PO_4, 0.12% (w/v) $(NH_4)_2SO_4$. Autoclave 30 min at 110°C.
7. Regeneration medium: 1% (w/v) Bacto peptone, 1% (w/v) Bacto yeast extract, 2% (w/v) galactose, 1 *M* sorbitol, 0.1% (w/v) KH_2PO_4, 0.12% (w/v) $(NH_4)_2SO_4$. Autoclave 30 min at 110°C.
8. Sorbitol-cycloheximide ice cube: 1 *M* sorbitol, 200 µg/mL cycloheximide. Should be prepared the day before the mitochondria isolation and stored at −20°C.
9. Cycloheximide solution: 100 mg/mL cycloheximide in ethanol solvent. Prepare 1 mL the day of mitochondria isolation; store at 4°C before use. Should not be conserved more than 1 d.
10. Sorbitol-cycloheximide buffer: prepare 1 *M* sorbitol stock solution. Autoclave 30 min at 110°C. Store at room temperature. Just before use, add 200 µg/mL cycloheximide to 100 mL 1 *M* sorbitol.
11. Mannitol buffer: 0.6 *M* mannitol, 30 m*M* Tris-HCl, pH 7.6, 5 m*M* MgAc, 100 m*M* KCl. Autoclave 30 min at 110°C. Store at room temperature. Just before use, complete with 5 m*M* β-mercaptoethanol, 200 µg/mL cycloheximide, 500 µg/mL heparin, and 1 L for 20 g of yeast dry weight of protease inhibitors (Sigma).
12. 30 mL Thomas Glass Potter with striated tip for more efficient cell breaking.

2.2. RNA Purification for Quantitative Polymerase Chain Reaction and Microarrays

1. TES buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* ethylenediaminetetraacetic acid, 0.5% (v/v) sodium dodecyl sulfate (SDS). Autoclave 30 min at 110°C. Store at room temperature.
2. Phenol-chloroform mix: phenol:chloroform 5:1. Store at 4°C.
3. Ready Red. Store at 4°C.
4. Sodium acetate: 3 *M* NaAc, pH 5.3. Autoclave 30 min at 110°C. Store at room temperature.
5. RNA purification kit: NucleoSpin RNA II kit from Macherey-Nagel.
6. Water: use molecular biology water for good RNA stability.

2.3. Quantitative Reverse Transcriptase Polymerase Chain Reaction

1. Polymerase chain reaction (PCR) machine (Mastercycler Eppendorf) is used for incubation steps.
2. Primers: random hexamers from Roche (1034731) and oligo dT from Invitrogen (yo1212). Store at −20°C.
3. Reverse transcriptase (RT) primer mix: 5 µg random hexamers (2.5 µL), 2 µg oligo dT (4 µL). Complete with ribonuclease (RNase)-free water to a final volume of 23 µL. Prepare on ice just before use.
4. BRL Superscript II kit (Gibco): this kit contains 5X SSII buffer, Superscript enzyme, and dithiothreitol (DTT) (0.1 *M*). Store at −20°C.

5. Deoxynucleotide 5′-triphosphate (dNTP) mix: 2.5 m*M* deoxyadenosine 5′-triphosphate, 2.5 m*M* deoxythymidine 5′-triphosphate, 2.5 m*M* deoxycytidine 5′-triphosphate, 2.5 m*M* deoxyguanosine 5′-triphosphate. Store at –20°C.
6. Reaction mix: 8 µL SSII buffer, 4 µL DTT, 2 µL dNTP. Prepare on ice just before use.
7. PCR extract kit: for retrotranscription product purification, use Nucleospin Extract Kit (Macherey-Nagel).
8. LightCycler instrument (Roche, 2011468).
9. LightCycler capillaries (20 µL) (Roche, 1909339).
10. Barrier tips (neptum) should be used to avoid deoxyribonucleic acid (DNA) contamination from pipetman.
11. QuantiTech Sybr Green PCR kit (Qiagen, 204143).
12. Primers: for every target transcript, a primer pair (for and rev primers) should be designed following kit instruction. Each primer is dissolved to a final concentration of 100 pmol/µL (10X primer). For and rev primers are then mixed to a final concentration of 10 pmol/µL (quantitative PCR [Q-PCR] primer mix). 10X primers and primer mix are stored at –20°C. Three types of primer pairs are used for precise quantification of RNA transcript localization: target transcript primers are used to quantify RNA of interest in each fraction, normalization primers are used to quantify mitochondrial RNA (e.g., *COX1* and *COX2*) and determine mitochondria purification yield, and contamination marker primers (e.g., *ACT1* and *RPL10*) are used to evaluate cytosolic RNA contamination.

2.4. Labeled Complementary DNA Synthesis for Microarray Analyses

1. Mastercycler personal (Eppendorf).
2. Random hexamers and oligo dT (12–18) (Invitrogen).
3. Molecular biology-grade water.
4. Superscript II RNase H reverse transcriptase, RT 5X first-strand buffer, and 0.1 *M* DTT (Invitrogen).
5. Cy3- and Cy5-linked deoxyuridine triphosphate (dUTP) from Amersham.
6. dNTP set, 100 m*M*.

2.5. RNA Hydrolysis Before Complementary DNA Purification

1. Pure NaOH.
2. 37% HCl.

2.6. Purification of Labeled Complementary DNA for Microarray Hybridization

1. 2K15 centrifuge (Sigma).
2. 5415 D centrifuge (Eppendorf).
3. 95% ethanol, spectrophotometry grade.
4. Qiaquick PCR purification kit.
5. 3 *M* Na acetate, pH 5.2.

2.7. Prehybridization of Microarrays

1. Yeast open reading frame microarray from the Service de Genomique du Departement de Biologie (www.transcriptome.ens.fr): about 6000 oligonucleotides representing the complete set of yeast open reading frames, deposited in duplicates on Ultragaps aminosilane slides (Corning).
2. 20X Standard Saline Citrate (SSC) from Qbiogen.
3. 20% (w/v) SDS.
4. 30% (w/v) bovine serum albumin (BSA).
5. Isopropanol (Merck).
6. 50-mL polypropylene tubes.

2.8. Microarray Hybridization

1. ArrayIT hybridization chamber (Telechem).
2. HS60 (60X, 22-mm) coverslips (Grace Biolabs).
3. 2X hybridization buffer: 50% (w/v) formamide, 10X SSC, 0.2% (w/v) SDS.
4. 70°C heating block.
5. 42°C water bath.

2.9. Microarray Washing

1. Washing buffer 1: 1X SSC, 0.2% (w/v) SDS.
2. Washing buffer 2: 0.1X SSC, 0.2% (w/v) SDS.
3. Washing buffers 3 and 4: 0.1X SSC.
4. CR412 centrifuge (Jouan) for 50-mL tube centrifugation.

2.10. Microarray Scanning and Image Analysis

1. Genepix 4000B scanner (Axon).
2. Genepix Pro 5.1 software.
3. PC Dell Dimension 8250, 42.4-GHz Pentium, 1-GB RAM, 75.50-GB hard drive; Windows 2000 or Windows XP.
4. Excel software.

2.11. Fixation of Cells and Spheroplasting for Fluorescent In Situ Hybridization

1. Buffer B: 1.2 *M* sorbitol (from 3 *M* autoclaved solution), 0.1 *M* potassium phosphate, pH 7.5 (from 1 *M* autoclaved solution); store at room temperature.
2. Formaldehyde, electron microscopy grade (Electron Microscopy Science, Fort Washington, PA).
3. Spheroplast buffer, 28.6 m*M* β-mercaptoethanol (Sigma, St. Louis, MO), 20 m*M* vanidyl ribonucleoside complex (New England Biolabs, Beverly, MA), 120 U/mL RNase inhibitor (Roche, Indianapolis, IN); in buffer B; prepare fresh.
4. Lyticase stock (Roche, Indianapolis, IN): 25,000 U/mL in water; store at –20°C.
5. 70% ethanol.
6. 22 × 22 mm type 1 coverslips (Fisher).

7. 0.1 *N* hydrochloric acid; dilute fresh.
8. 0.01% (w/v) poly-L-lysine (Sigma); dilute fresh.

2.12. Probe Synthesis Labeling and Purification for Fluorescent In Situ Hybridization

1. Amino-modifier C6 dT (Glen Research, Sterling, VA).
2. Synthesis is performed on an Applied Biosystems automated DNA/RNA synthesizer (model 392/394) using a 0.2-µm scale cartridge.
3. Fluorophores typically used for labeling are fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR), Cy3, Cy3.5, Cy5, Cy5.5 Fluorolink™ monofunctional dye (Amersham Biosciences, Piscataway, NJ).
4. Carbonate buffer: 0.1 *M* sodium carbonate, pH 9.0; store frozen at –20°C in 500-µL aliquots.
5. Sephadex G50 (Sigma), rehydrated and degassed in 10 m*M* TEAB (*see* **item 6**) and packed into a 25-mL plastic pipet by gravity flow.
6. 2 *M* Triethylamine bubbled (TEAB): weigh 101 g triethyl amine (Sigma) into a flask, add 200 mL water, and insert a Pasteur pipet in the solution connected to a dry ice chamber. Allow the Pasteur pipet to bubble overnight in the solution to verify that the pH is below 8.0. Adjust volume to 500 mL and store at 4°C. Triethyl amine is extremely corrosive and should not be exposed to plastic when pure; the bubbling operation should be conducted under a fume hood.
7. 10 m*M* TEAB: dilute from 2 *M* TEAB and store at 4°C.

2.13. Fluorescent In Situ Hybridization

1. 2X SSC: dilute from 20X SSC (Roche).
2. Phosphate-buffered saline (PBS): dilute from 10X PBS (Roche).
3. Competitor nucleic acids mix: 2.5 mg/mL sonicated salmon sperm DNA (Sigma), 2.5 mg/mL *Escherichia coli* transfer RNA (Sigma); store at –20°C in 100-µL aliquots.
4. Formamide solution: 40% (w/v) formamide (Sigma) in 2X SSC.
5. Solution F: 80% (w/v) formamide, 10 m*M* sodium phosphate, pH 7.0.
6. Solution H: 4X SSC, 4 µg/µL RNase-free BSA (Roche), 20 m*M* vanidyl ribonucleoside complex, 0.24 U/µL RNase inhibitor.
7. Triton wash solution: 0.1% (v/v) Triton X-100 in 2X SSC.
8. 4,6-Diamidino-2-phenylindole (DAPI) solution: 0.5 µg/mL DAPI (Molecular Probes) in PBS; store at 4°C.
9. Mounting medium: ProLong Antifade Kit (Molecular Probes).
10. Nail polish.

3. Methods

The following three methods aim at assessing the spatial distribution of nuclear-encoded mRNAs coding for mitochondrially localized products. The first two methods, quantitative reverse transcriptase polymerase chain reaction

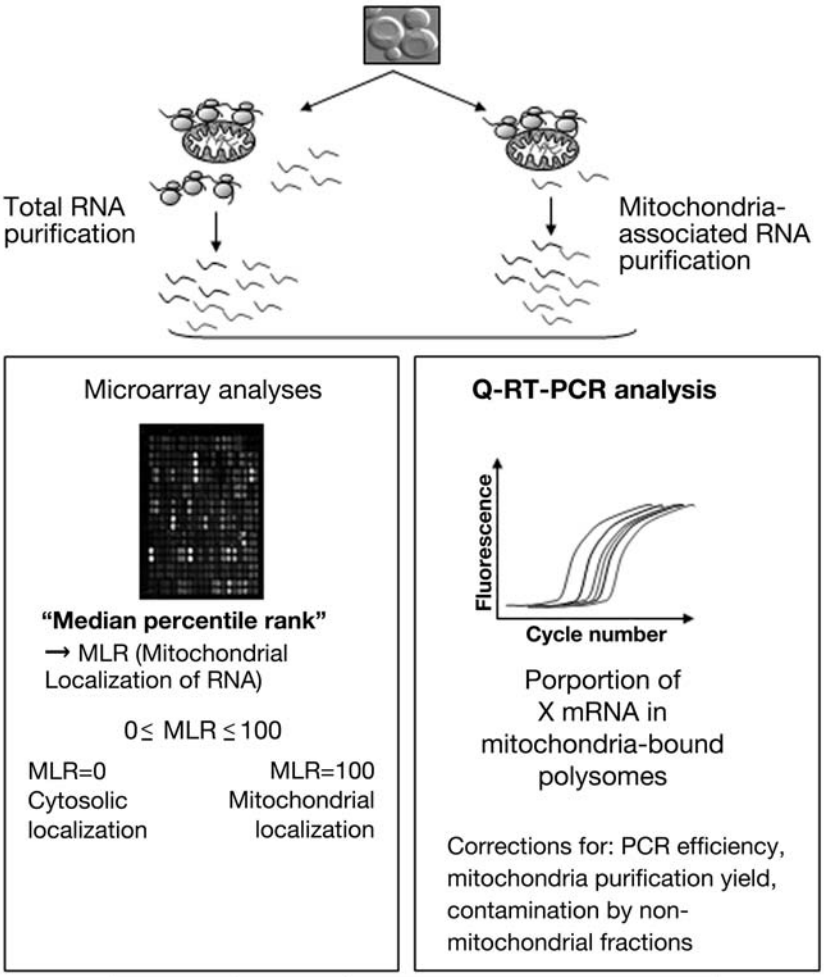
(Q-RT-PCR) and microarray analyses, rely on the biochemical purification of the mitochondria-bound polysomes, whereas the third method allows observation of the in vivo localization of specific mRNAs. The aim of the real-time Q-RT-PCR analysis (**Subheadings 3.2.** to **3.3.**) is to provide a quantitative assessment of the spatial distribution of a specific mRNA based on a biochemical purification of mitochondria-bound polysomes (*see* **Note 1**, **Fig. 1**). The genomewide approaches using DNA microarrays (**Subheadings 3.4.–3.10.**), although less precise, have the clear advantage of allowing global analyses of RNA subpopulations. Therefore, microarray analyses identify subgroups of colocalized RNA and allow searching for correlations between mRNA location and protein properties or characterization of new mRNAs located to the mitochondria and likely to encode mitochondrial proteins ***(6)***. The microarray protocol used for mRNA mitochondrial location analyses is a standard one, identical to the protocols used for gene expression studies. However, the methods used for data analyses are different (*see* **Note 2**, **Fig. 2**).

Finally, single-cell fluorescent *in situ* hybridization (FISH) experiments (**Subheadings 3.11.–3.15.**) represent a necessary in vivo complement of the two preceding methods. Messenger ribonucleoproteins (mRNP) cytoplasmic localization can be directly observed by fluorescence microscopy, either in live cells using green fluorescent protein reporter proteins ***(9)*** or in fixed cells where endogenous mRNAs can be detected by FISH. New developments in probe design and in fluorophore chemistry allow routine detection of single molecules of mRNA within their cellular environment ***(10,11)***. FISH is particularly well suited or dissecting the different mechanisms governing mRNA localization in yeast ***(12)***.

The protocol we present, adapted from **refs. *13*** and ***14***, is designed to simultaneously compare the special distribution of different mRNAs relative to each other or to the mitochondria. FISH is particularly adapted in this case because mitochondria can be unambiguously detected using probes directed to the mitochondrial ribosomal RNAs (rRNAs), allowing for the simultaneous detection of mRNAs and mitochondrion in a single step. As an example, we show the simultaneous detection of the mitochondria-addressed *ATP2* mRNA compared to the *YRA1* mRNA, which is used as a nonlocalized control.

3.1. Mitochondria-Bound and Total RNA Isolation for Q-PCR and Microarrays

1. Preculture: CW252 yeast strain is grown in 20 mL YPGal at 30°C for 24 h with agitation (250 rpm).
2. Culture: an appropriate aliquot of preculture is transferred in 1 L Gal-rich medium and incubated for 1 night at 30°C with agitation; the next morning the OD_{600} should be between 0.8 and 3 (CW252 generation time in Gal-rich medium is around 2.5 h). The OD_{600} must be properly measured before mitochondria



Gene	MLR	% mRNA in mitochondria-bound polysomes	
		Before Correction	After Correction
ACT1	24	17%	0%
ATP2	100	59%	50%
ATP3	90	49%	38%
COX4	27	7%	0%
COX6	30	11%	0%

Fig. 1. Biochemical methods to analyze the spatial distribution of mRNAs coding for proteins imported into mitochondria and example of results for *ACT1* (contamination marker), *ATP2*, *ATP3* (mitochondria-associated RNA), *COX 4*, and *COX6* (non-mitochondria-associated RNA).

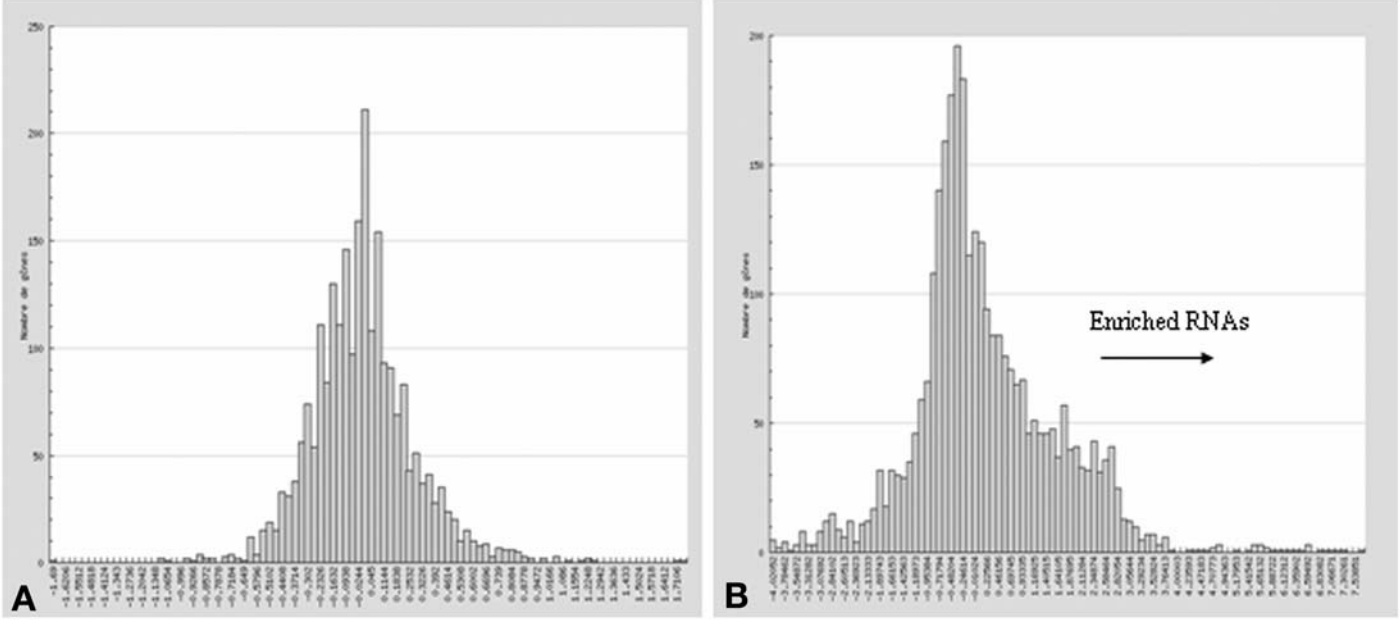


Fig. 2. The distribution of mitochondrial location analyses can be skewed. (**A**) Example of distribution of the Cy5/Cy3 fluorescence ratios (Rf) that can be obtained from a standard global gene expression experiment. Both repressed and activated genes are expected; the distribution is similar to a normal distribution. (**B**) Example of distribution of Rf that can be obtained from microarray experiments comparing total RNAs and mitochondria-associated RNAs. Only RNAs enriched in the Cy5 channel are expected: the distribution is skewed by enriched RNA.

isolation to evaluate dry weight (DW) using the formula $DW = 0.28 \times OD_{600} \times V$ (V is the volume in liters).

3. Cells are collected by centrifugation for 15 min at 4300*g* at 4°C and washed with water stored at 4°C. Note that all centrifugation steps allowing washing and changing culture medium are performed for 15 min at 4300*g* at 4°C.
4. The cells are suspended in 20 mL per gram dry weight of preincubation buffer and then incubated in a 200-mL Erlenmeyer flask at 30°C with agitation for 10 min. Cells are then washed several times with 20 m*M* potassium phosphate buffer, pH 7.4; a total volume of 1 L buffer is used for this washing step.
5. Cells are suspended in 1-L Erlenmeyer flask to a final OD_{600} of 12 in digestion buffer. 5 mg zymolyase is added to the culture, and the mix is incubated for 10 min with agitation for enzymatic digestion of cell walls. After incubation, digestion should be performed at 80%. To verify digestion efficiency, the decrease of OD_{600} can be measured by mixing 50 µL culture in 1 mL water. Vigorously shake before measuring OD_{600} to perform cell lysis by osmotic shock in water. After digestion, wash cells with washing medium and then incubate in 200 mL regeneration medium in 1-L Erlenmeyer flask at 30°C with gentle agitation for 3 h.
6. During the incubation step, prepare cycloheximide solution and weigh 20 mg heparin, which will be added to 40 mL mannitol buffer just before use (*see* **step 9**).
7. Add 600 µL cycloheximide solution to the culture to block translation machinery and incubate at 30°C with agitation for 10 min additional. Set apart 8 mL culture, which will be used to prepare total RNA, dispense them in 2-mL Eppendorf tubes, and centrifuge at 18,000*g* at 4°C for 3 min. Wash pellet with 1 *M* sorbitol. Store the cell pellet at −80°C.
8. Perform a thermal shock to stop cell metabolism by dispensing culture in a beaker containing sorbitol-cycloheximide ice cubes. From that moment, all steps must be performed at 4°C in a cold room. Centrifuge culture at 4300*g* for 15 min at 4°C and wash with 100 mL sorbitol-cycloheximide buffer.
9. Suspend the pellet in 4 mL mannitol buffer. Cells are broken by 20 strokes in a glass potter, transferred into a 50-mL falcon tube, and centrifuged 8 min at 1700*g* to remove nucleus. Supernatant is taken off and transferred in a new Falcon. The pellet is suspended once more in 3 mL mannitol buffer, submitted to 20 strokes in the glass potter, and again centrifuged at 1700*g* for 8 min. The supernatant is added to the previous one, and a last step of centrifugation allows complete nuclear removal. Supernatant is taken off and transferred into a 15-mL tube that fits in high-speed centrifuge adapters. Centrifugation at 14,600*g* for 30 min leads to a red pellet of mitochondria, which is washed one time with mannitol buffer before storing at −80°C.

3.2. RNA Purification for Q-PCR and Microarrays

1. Suspend cells and mitochondrial pellets in 400 µL TES buffer and transfer in 1.5-mL Eppendorf tubes. Add 400 µL phenol-chloroform mix and incubate 15 min at 65°C. During the incubation step, vortex tubes 30 s every 5 min to homogenize. Centrifuge at room temperature for 15 min at 18,000*g*.

2. Transfer the aqueous phase in a new 1.5-mL Eppendorf tube and add 400 µL phenol-chloroform mix. Vortex three times for 30 s; centrifuge 15 min at 18,000*g*.
3. Transfer the aqueous phase in a new 1.5-mL Eppendorf tube and add 300 µL chloroform. Vortex three times for 20 s; centrifuge 2 min at 18,000*g*.
4. Transfer aqueous phase in tubes containing 30 µL sodium acetate, add 600 µL ethanol, and incubate 1 h at −20°C for RNA precipitation. Centrifuge 15 min at 18,000*g* at 4°C and resuspend the pellet in 20 µL RNase-free water.
5. Quantify RNA by measuring 260-nm absorbance (1 absorbance unit corresponds to 40 µg/µL RNA); about 250 µg total RNA purification and 50 µg from mitochondria purification are obtained in routine experiments.
6. Perform a purification step using the RNA purification kit. The RNA cleanup is an essential step for the quality of the reverse transcription. Follow the recommendations of the Macherey-Nagel kit (total RNA preparation from biological fluids section). Elute the column with 60 µL water first and then reelute with the same 60 µL.
7. Quantify purified RNA. For Q-RT-PCR, adjust concentration to 50 ng/µL. Store RNA at −20°C. When used, always keep RNA tubes in ice to avoid degradation.

3.3. Quantitative RT-PCR

1. Retrotransciption: for each sample, add 1 µL RNA to 23 µL RT primer mix. Incubate for 10 min at 70°C. Put on ice and add 14 µL reaction mix and 2 µL Superscript polymerase. Incubate 10 min at 23°C, then 1 h at 42°C. Store at −20°C. It is recommended to perform a control reaction without added RNA to verify the absence of contamination in the RT mix.
2. Purification: purification of RT product is an important step for Q-PCR efficiency. Just follow the recommendations of the Macherey-Nagel PCR extract kit (protocol for direct purification of PCR products). Elute with 50 µL elution buffer, then dilute to 1/10, 1/50, and 1/100 for Q-PCR.
3. Real-time Q-PCR: for each sample dilution (1/10, 1/50, 1/100), mix in a capillary tube 10 µL 2X Sibr mix and 8 µL H_2O (from QuantiTech Sybr Green PCR kit), 1 µL RT dilution, and 1 µL desired Q-PCR primer mix. Perform a LightCycler program as follows: a first step of initial polymerase activation of 15 min at 95°C, a second step of 55 cycles of amplification that consists of 15 s at 95°C (denaturation step), 30 s at 54°C (annealing step), and 20 s at 72°C (extension step). Transition rate is 20°C/s. Fluorescence acquisition is performed during the extension step. After amplification, the cycler performs a melting curve of product by increasing slowly (0.1°C/s) from a low temperature (65°C) to a high temperature (95°C) and measuring the decrease in the fluorescence. This allows verification of the amplification of a unique and specific product during Q-PCR. The program ends with a cooling step (20°C/s) to reach 40°C. Do not forget to carry out a Q-PCR with purified RNA diluted to 1/10 as a template to check the absence of DNA in RNA purifications.
4. Q-PCR data analysis is conducted as indicated in **Note 1**.

3.4. Labeled Complementary DNA Synthesis for Microarray Analyses

1. Prepare two 0.2-mL PCR tubes containing about 10 µg of either mitochondria-bound or total RNAs.
2. Add 5 µg random hexamers and 2 µg oligo dT in each tube. Add water to a final volume of 23 µL.
3. Incubate the tubes at 70°C for 10 min in a Mastercycler PCR machine.
4. While the tubes are incubating, prepare the following mixture: 16 µL RT 5X first-strand buffer plus 8 µL DTT 0.1 *M*, 4 µL of dNTP mix, and 4 µL Superscript II 200 U/µL enzyme.
5. Put the PCR tube on ice. Add 1 µL Cy3-dUTP 1 m*M* in one of the tubes. Add 1 µL Cy5-dUTP 1 m*M* in the other tube. When reproducing experiments, make a dye swap (inverse the labeling). Add 16 µL of mix to each tube.
6. Leave the tubes 5 min at room temperature.
7. Incubate for 2 h at 42°C in a Mastercycler PCR machine.

3.5. RNA Hydrolysis Before Complementary DNA Purification

1. Add 15 µL 0.1 *M* NaOH.
2. Incubate for 10 min at 70°C in the Mastercycler.
3. Neutralize the pH by adding 15 µL 0.1 *M* HCl.

3.6. Purification of Labeled Complementary DNA for Microarray Hybridization

1. Pool the two PCR tubes in 1 Eppendorf centrifuge tube. Add 1/10 volume 3 *M* Na acetate and 2.5 volumes ethanol.
2. Precipitate 30 min at −80°C. Centrifuge 30 min at 4°C at 18,000*g*. Discard supernatant. Resuspend pellet in 40 µL water and add 4 µL 3 *M* Na acetate.
3. Add 200 µL Qiagen PB buffer. Load sample to a Quiaquick PCR purification column and centrifuge at 13,500*g* for 1 min at room temperature in the 5415 D centrifuge.
4. Remove liquid, add 600 µL PE buffer, and centrifuge for 1 min. Remove liquid and centrifuge 1 min more to dry the column. Remove liquid. Place the column in a new centrifuge tube. Add 30 µL of water prewarmed at 42°C. Centrifuge 1 min. Keep the eluate for hybridization step.

3.7. Prehybridization of Microarrays

This treatment aims at inactivating the free aminosilane groups before hybridization to avoid nonspecific interaction of the labeled complementary DNA (cDNA) with the slide. It can be done during the 2-h incubation left during the labeling reaction.

1. Preheat 5X SSC, 0.1% (w/v) SDS, 1% (w/v) BSA mix in a Falcon tube at 42°C.
2. Place the slide in the Falcon tube and incubate for 45 min.
3. Rinse the slides five times (30 bottom-up moves) in five 50-mL Falcon tubes filled with water.

4. Put the slide in isopropanol and leave it to dry on a paper towel. Avoid any contact between the DNA spots (opposite to the serial number) and the paper. Stock the slide in a plastic box protected from light and moisture. Use prehybridized slides within 12 h.

3.8. Microarray Hybridization

1. Preheat the purified cDNA and hybridization buffer at 70°C for 3 min.
2. Prepare the hybridization chamber by putting 40 µL water in the holes and place the slide into the chamber (on yeast microarrays, the DNA spots are opposite the serial number label).
3. Add 35 µL hybridization buffer to the 30 µL purified cDNA. Put this mix onto the slide (avoiding bubbles as much as possible). Put the on coverslip carefully to make the liquid spread as homogeneously as possible (heterogeneity in the hybridization may lead to local heterogeneity in the final signal).
4. Close the hybridization chamber, drop it in a water bath at 42°C, and leave it to incubate overnight.

3.9. Microarray Washing

1. Get the chamber out of the water bath and dry it with a paper towel (*keep it horizontal*). Take the slide and move it up and down in a 50-mL Falcon tube filled with washing buffer 1 prewarmed at 42°C to make the coverslip fall down. Put the DNA spot side opposite the coverslip and drop it in the Falcon tube. Move the Falcon bottom-up about 30 times to rinse the slide.
2. Proceed the same in washing buffers 2–4 at room temperature.
3. Centrifuge the slides 5 min at 500*g* at room temperature in a new, clean, empty Falcon tube. The slide should not dry before the centrifugation step, so put the slide directly from washing buffer 4 into the centrifuge in which you have put the empty Falcon tube. Do not close the tube to avoid condensation. After centrifugation, slides are ready for scanning.

3.10. Microarray Scanning and Image Analysis

1. Scan the slide with the Genepix 4000B following the Axon's instructions. Use the "histogram" window to get the distribution of the signal in Cy3 (green) and Cy5 (red) channels. Look at the percentage of saturating pixels and dynamic range in each channel. Set up the Photomultiplicator voltage so that you have no saturating spots while using most of the dynamic range of fluorescence measurements (2^{16} values).
2. For image analyses, use the Genepix Pro software recommendations. The image analysis is rather automatic. Just control that the localization of the spots is correct and flag as "bad" the spots in which signal is obviously caused by artifacts (dusts, slide damage, etc.) rather than DNA hybridization. Get the "result" *.gpr file for further analysis of the data.
3. For microarray data, analysis *see* **Note 2**.

3.11. Fixation of Cells and Spheroplasting for FISH

1. Cells are grown in appropriate conditions (medium and temperature according to the strain used) to early log phase in 50 mL medium with shaking.
2. Cells are fixed 45 min at room temperature with occasional shaking by addition of the formaldehyde directly to the culture to a final concentration of 4%.
3. Cells are pelleted at 3000g and washed three times with 10 mL ice-cold buffer B.
4. Cell pellet is resuspended by gentle pipeting in 750 mL spheroplast buffer in a 1.5-mL tube; 24 µL lyticase stock solution are added, and cells are incubated at 30°C for 5–20 min with occasional inversion (the incubation time needs to be optimized according to the strain used).
5. Cells are pelleted 4 min at 3500*g* and 4°C, washed once with 1 mL buffer B, and resuspended in 750 µL to 1.4 mL buffer B depending on the desired concentration of cells for imaging.
6. Spread 100 µL cells with the tip of a pipet on a poly-L-lysine-treated cover slip (in the six-well tissue culture plate) and incubated at 4°C for 30 min (vibrations can prevent cells from adhering and should be avoided at this step).
7. Slowly add 3 mL buffer B to the well (avoiding direct flow).
8. Buffer B is replaced by 70% ethanol. Cells need to stay in 70% ethanol for at least 15 min at −20°C and can be kept for weeks under these conditions, sealing the plate with parafilm to avoid ethanol evaporation.

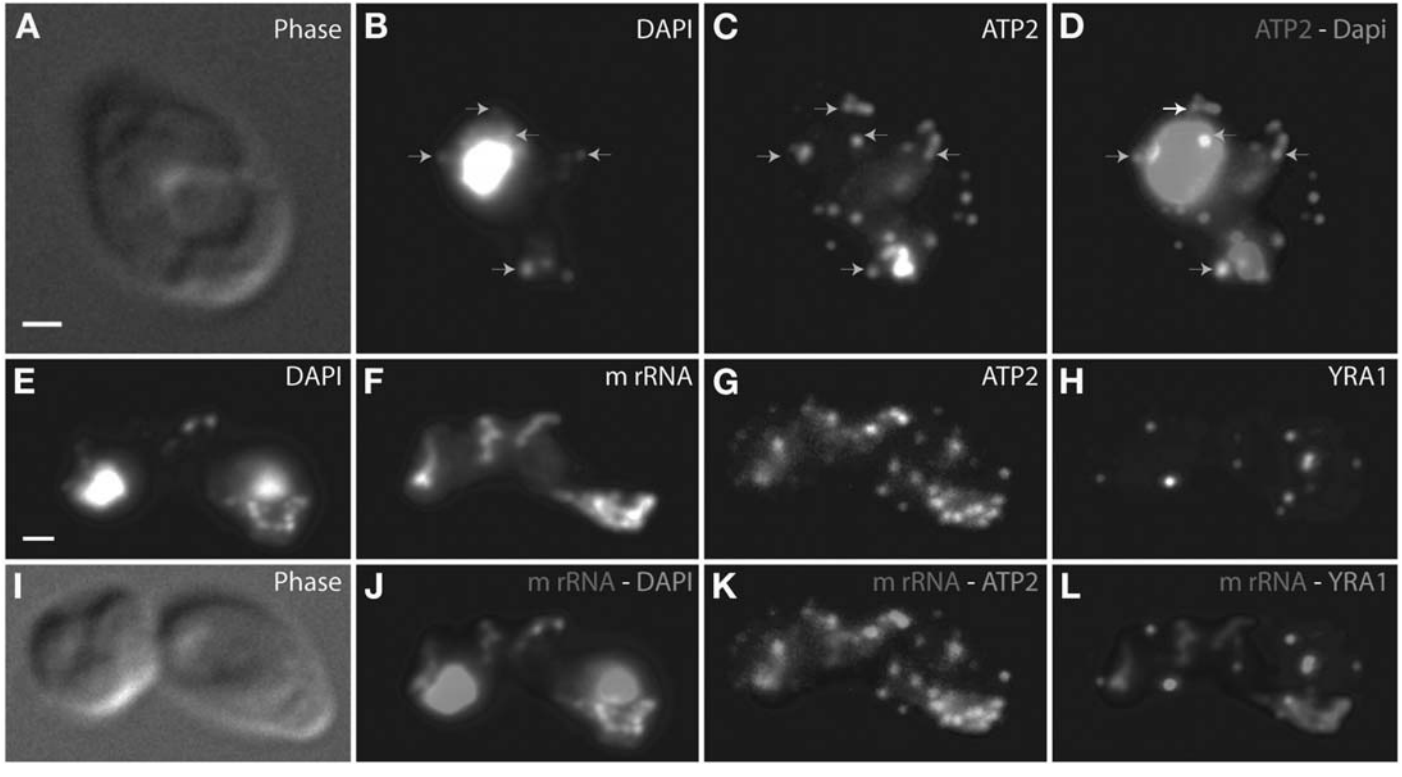
3.12. Probe Synthesis Labeling and Purification for FISH

1. Typically 4–6 antisense oligonucleotides of 50 nucleotides each are selected. Probes used in the same hybridization should have the same GC content (the protocol as described is optimized for 50% GC 50 nucleotides oligonucleotides). Probes with a low self-annealing potential are favored. Probe selection is facilitated by the Oligo6 software (Molecular Biology Insights, Cascade, CO). Five thymidines from the sequence of the oligonucleotide are replaced by aminoallyl thymidines, respecting spacing of at least eight nucleotides between each to avoid quenching. Probes used in **Fig. 3** are described in **Table 1**.
2. Probes are synthesized on an Applied Biosystems automated DNA/RNA synthesizer (model 392/394) according to manufacturer recommendations.
3. Probes are purified on oligonucleotide purification cartridge (OPC) columns (Applied Biosystems, Foster City, CA) according to manufacturer instructions.
4. Pure probes are dehydrated and resuspended in 100 µL water, and their concentration is determined by ultraviolet (UV) spectrophotometry. Dry 5–20 µg probe and use for labeling.
5. Cyanine probes (1 dry aliquot) are resuspended in 20 µL carbonate buffer. The total amount of resuspended dye is used to resuspend the dried probe by pipeting and vortexing. FITC probes need to be resuspended in dimethyl sulfoxide, and the labeling volume needs to be increased not to exceed 20% dimethyl sulfoxide. Labeling reaction is kept in the dark at room temperature for at least 12 h.

6. The probe is diluted with 50 µL 10 m*M* TEAB and loaded onto the Sephadex G50 column that is running 10 m*M* TEAB by gravity flow. At this point, the labeled probe runs faster than the free dye and should be visible as a faint band compared to the intensity of the free dye. (FITC dyes are best followed under short UV illumination.) The probe is collected in 1.5-mL tubes (typically 2.5–4 mL) and dehydrated.
7. Probe pellets are pooled and resuspended in 100 µL water; DNA concentration as well as labeling efficiency are determined by spectrophotometry (typically, 60–95% labeling efficiency is obtained). Probes are diluted to 40 ng/µL with TE and stored at −20°C.

3.13. Fluorescent In Situ *Hybridization*

1. Place 1 box of type 1 coverslips that are 22 × 22 mm in a beaker containing 250 mL 0.1 *N* hydrochloric acid and boil for 20 min.
2. Abundantly rinse coverslips with distilled water to remove any traces of acid; autoclave and store at 4°C immersed in water (dried coverslips stick to each other and are difficult to manipulate).
3. Individual coverslips are placed on Whatman paper and 100 µL poly-L-lysine solution is applied and spread with a pipet tip on each coverslip; after 2 min, the poly-L-lysine is removed, and the coverslips are air dried for 3 h.
4. Each coverslip is placed on a six-well plate (treated side up) and washed three times for 10 min with 3 mL water.
5. Water is removed, and coverslips are rested at a 45° angle on the wall of the wells (this will prevent them from sticking to the bottom of the plate) and allowed to dry to completion.
6. Dry coverslips are rested in the bottom of the wells and can be stored at room temperature.
7. Combine 2–10 ng of each individual probe (not exceeding 50 ng total) in a 1.5-mL tube with 4 µL competitor nucleic acids mix and dry under vacuum.
8. While the probes are drying, rehydrate cells twice for 5 min at room temperature in 2X SSC and 5 min in formamide solution.
9. Resuspend the probes in 12 µL solution F and heat at 100°C for 3 min.
10. Add 12 µL solution H and mix; 20 µL of the mix are dropped on the bottom of a Petri dish "hybridization chamber." Place 1 coverslip on the drop (cells face down). Fill the cap of a 50-mL tube with formamide solution and place in the dish to ensure humidification. Seal the Petri dish with parafilm and incubate for a minimum of 3 h (optimal after 10 h) at 37°C.
11. Remove coverslips, place back in six-well tissue plates, and wash twice for 15 min at 37°C with prewarmed formamide solution.
12. Wash coverslips for 15 min with 0.1% Triton X-100 solution with gentle shaking at room temperature.
13. Wash coverslips twice for 15 min with 1X SSC with gentle shaking at room temperature.
14. Incubate coverslips 5 min in 1 mL DAPI solution at room temperature.
15. Wash coverslips twice for 5 min with 2 mL PBS at room temperature.


A
Phase
B
DAPI
C
ATP2
D
ATP2 - Dapi
E
DAPI
F
m rRNA
G
ATP2
H
YRA1
I
Phase
J
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m rRNA - ATP2
L
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Fig. 3.

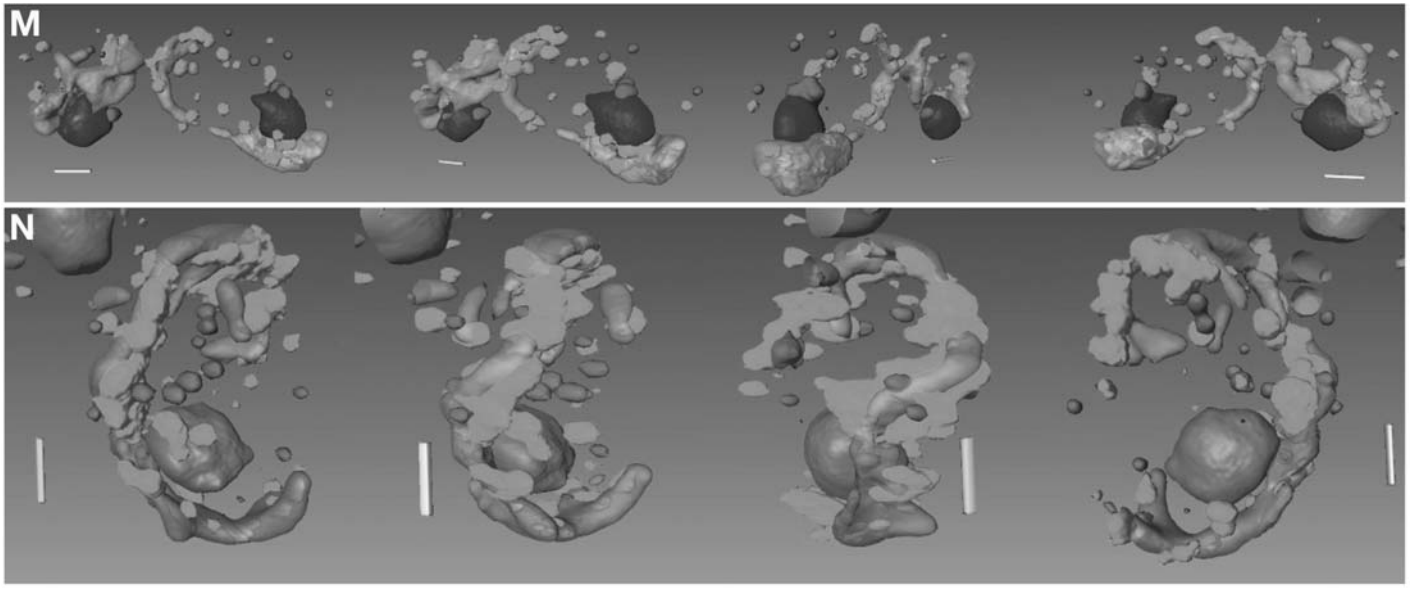

Fig. 3. Mitochondria and localization of mRNA molecules. (**A**)–(**D**) Single-plane distribution of *ATP2* mRNA: (**A**) Phase; (**B**) nucleus and mitochondria detected by DAPI staining; (**C**) *ATP2* mRNA detected by FISH using Cy3-labeled probes. Arrows show the position of DAPI-stained cytoplasmic structures that can be difficult to discriminate from the very bright nucleus. (**E**)–(**M**) simultaneous detection of (**E**) DAPI, (**F**) the mitochondrial rRNA (Cy3.5), (**G**) *ATP2* (Cy5), and (**H**) *YRA1* (Cy3). Three consecutive planes were merged. The simultaneous detection of three different fluorophores requires the use of narrow-band filters, resulting in a noticeable diminution of the signal-to-noise ratios (compare **G** and **C**) (**I**) phase; (**J**) merge, DAPI in light gray (green) from **E** and mitochondrial rRNA from **F**; (**K**) merge mitochondrial rRNA in dark grey (red) from **F** and *ATP2* mRNA in light gray (green) from **G**; (**L**) merge mitochondrial rRNA in dark gray (red) from **F** and *YRA1* mRNA in light gray (green) from **H**; (**M**) 3D representation of the cellular volume (same cells as in **E**–**L**) *ATP2* mRNA and mitochondrial rRNA are made green (Cy5) and purple (Cy3.5), respectively, and they appear as elongated intermingled gray structures. *YRA1* mRNA can be observed in red (Cy3); it appears as round, single, gray structures. (**N**) Same as **M** showing the variability of *ATP2* addressing from cell to cell (compare **M** and **N**). Scale bar: 1 μm.

Table 1
Probes Used

ATP2-1	**t**ACCAGTG**T**CAAGAACCTTT**T**CACCACGGACCAAACCT**T**C GGTACCA**T**CCA
ATP2-2	**t**ACCAACACCGG**T**GAAAACGGAAAAACCACCA**T**GGGCCT TGGCGATAT**T**GT
ATP2-3	A**T**ATTCAGCGA**T**CGTCAAACCAG**T**TAAAGCGACTC**T**GGC TCTGGCTCC**T**G
ATP2-4	**T**GGCAGGAGCAGGA**T**CTGTTAAATCA**T**CGGCTGGAACA**T** AAACGGCT**T**GC
ATP2-5	**t**GGCGACG**T**CATAATGTTCT**T**GACCGACAACGGCGGCA**T**C CAATAACCT**T**G
15S m rRNA	**t**AAACCATTA**T**GATTAACGC**T**CGCCCTCTTTG**T**GTTACCGC GACTGC**T**GGC
21S m rRNA-1	**t**GACCCGAAAGGGAACCGGAACCCCGAAGAGGGG**T**TCAC ACC**T**ATTAAAAA**t**a
21S m rRNA-2	AGC**T**GCATAGGGTC**T**TTCCGTCTTGC**T**GAAGGTACA**T**AGC ATCTTCAC**T**ACGAT

Aminoallyl-modified thymidines are bold; sequence complementary to the target gene is capitalized. An additional aminoallyl T was added 5′ or 3′ of ATP2-1, ATP2-2, ATP2-5, 15S mitochondrial rRNA, and 21S mitochondrial rRNA-1 to increase the labeling of the probes in which the sequence did not offer five optimally spaced thymidines.

16. Mount coverslips on a drop of mounting solution; remove excess solution with a kimwipe and seal coverslips with nail polish.
17. Protect coverslips from light; these can be kept at −20°C for years.
18. Perform imaging on an Olympus BX61 upright microscope using a 100 × 1.35-numerical aperture (NA) objective; illumination is provided by a 100-W mercury lamp.
19. Choose filter cubes to resolve spectrally the used fluorophores from each other (Chroma, Rockingham, VT) and perform test experiments in which only one fluorochrome is used to address possible leaks of the staining in the other used channel.
20. Perform three-dimensional (3D) sampling, acquiring 40 images spaced by 200 nm in the *z*-axis.
21. Perform image acquisition using IPlab (Schanalytics) and image processing using Image J (W. S. Rasban, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).
22. Typically, the detection of a particular RNA using five different antisense oligonucleotides (a total of 25 dye molecules in average) provides sufficient signal-to-noise ratio to ensure detection of single molecules. The diameter of mRNPs is below the resolution of the light microscope; therefore, molecules of mRNAs present in a radius range inferior to 200 nm are detected as single objects. The number of molecules present in a specific structure is proportional to the total fluorescence.

The mRNAs that cluster to the vicinity of mitochondria often cannot be resolved as individual molecules.

23. The mitochondrial network is a 3D structure, and images observed on single planes are difficult to interpret because mitochondria and their associated mRNAs can be seen in different planes. The visual inspection of the successive planes of the 3D stack is usually sufficient to detect mitochondrial association. For display purposes, individual planes can be selected (**Fig. 3A–D**). Several stacks can be combined using a maximum projection algorithm (**Fig. 3E–L**). A global view of the 3D cytoplasmic volume can be restored using software solutions such as Imaris (Bitplane, Exton, PA) or Amira (Mercury Computer Systems, San Diego, CA) offering a virtual representation of the volumes (**Fig. 3M,N**)

We are currently developing software solutions that would facilitate the automatic scoring of mRNPs in the vicinity of the mitochondria to establish genetic screens that allow us to dissect the molecular mechanism of mitochondrial mRNA addressing.

4. Notes

1. Q-PCR data analysis:
   Notations: In notations, *c* stands for cellular extract and *m* for mitochondrial extract.

Vm	Total volume of yeast culture used to mitochondrial isolation
Vc	Volume used for total RNA preparation
Qm	Quantity of RNA purified from mitochondrial isolation
Qc	Quantity of RNA purified from cells
Qpcr	Quantity of RNA used for Q-PCR experiment
$Q_c^{PCR}X$	PCR mix quantification of X RNA in total cellular RNA
$Q_m^{PCR}X$	PCR mix quantification of X RNA mitochondrial fraction
$Q_c^{T}X$	Total quantification of X RNA in total cellular RNA
$Q_m^{T}X$	Total quantification of X RNA in mitochondrial fraction
${}^{c}Q_m^{T}X$	Total quantification of X RNA in mitochondrial fraction after correction by contamination rate
$Q_{Cy}^{T}X$	Total quantification of X RNA in cytoplasm
$Rm(X)$	X RNA spatial distribution: percentage of RNA bound to mitochondria
Rc	Contamination rate
${}^{c}Rm(X)$	X RNA spatial distribution after correction by contamination rate
E	Q-PCR efficiency (i.e., number of DNA targets obtained after a PCR cycle from one target template; $0 \leq E \leq 2$)
K	Threshold for amplified DNA during Q-PCR used to determine the initial template quantity

Cpm(*X*) Mitochondrial threshold cycle for X RNA, the Q-PCR cycle at which the amplification plots reach the threshold in mitochondrial RNA

Cpc(*X*) Cellular threshold cycle for X RNA, the Q-PCR cycle at which the amplification plots reach the threshold in total RNA

Q-PCR fluorescence curve analyses: The PCR reaction profile can be divided in three steps: an early background phase, an exponential phase, and a plateau. During the exponential phase, the amplification course is described by the equation $Q_n = Q_0 \times E^n$, where Q_n is the amount of target at cycle n, Q_0 is the initial amount of target, and E is the efficiency of amplification.

To compare target initial amount in different samples, a threshold K for amplification is set. Cp is the corresponding cycle number required to correlate real-time fluorescence curves to initial template concentration according to the equation $K = Q_0 \times E^{cp}$.

Different methods are used to determine Cp; for review, see Randy Rasmussen's paper at http://www.idahotec.com/lightcycler_u/lectures/quantification_on_lc.htm. In the laboratory, we use the second derivative maximum method, for which no human decision is required to help the software find the exponential portion of the amplification.

Determination of Q-PCR Efficiency, E, for a given primer mix. The equation $K = Q_0 \times E^{cp}$ can be linearized to $\log(K) = \log(Q_0) + C_p \times \log(E)$. So, arranging the form gives the following standard curve equation:

$$Cp = -\frac{1}{\log(E)} \times \log(Q_0) + \frac{\log(K)}{\log(E)}$$

Instead of realizing an external standard curve using genomic DNA, results from the different RNA dilutions can be exploited to determine E. Considering the initial quantity of template in nondiluted RNA $Q_{0(1/1)}$ for each dilution:

$$Q_{0(1/d)} = \frac{Q_{0(1/1)}}{d}$$

and the standard curve equation can be modified as follows:

$$Cp = -\frac{1}{\log(E)} \times \log\left(\frac{1}{d}\right) - \frac{1}{\log(E)} \times \log(Q_{0(1/1)}) + \frac{\log(K)}{\log(E)}$$

$$Cp = -\frac{1}{\log(E)} \times \log\left(\frac{1}{d}\right) + b$$

So, the slope of the curve

$$Cp = f\left[\log\left(\frac{1}{d}\right)\right]$$

gives a direct assessment of Q-PCR efficiency for the studied target.

Determination of mRNA spatial distribution. Mitochondrial RNAs (*COX1, COX2*) are used to normalize Q-PCR results and determine mitochondrial purification yield η. This yield takes into consideration efficiency of different steps from biochemical purification to RT-PCR.

$$\eta = \frac{Q_m^T COX1}{Q_c^T COX1}$$

Considering the following equations:

$$Q_m^T COX1 = Q_m^{PCR} COX1 \times \frac{Qm}{Qpcr} \text{ and } Q_m^{PCR} COX1 = \frac{K}{E^{Cpm(cox1)}}$$

$$Q_c^T COX1 = Q_C^{PCR} COX1 \times \frac{Qc}{Qpcr} \times \frac{Vm}{Vc} \text{ and } Q_c^{PCR} COX1 = \frac{K}{E^{Cpc(cox1)}}$$

The purification yield is

$$\eta = \frac{E^{Cpc(cox1)}}{E^{Cpm(cox1)}} \times \frac{Qm}{Qc} \times \frac{Vc}{Vm}$$

This yield is used to determine mitochondrial spatial distribution for each RNA:

$$Rm(X) = \frac{Q_m^T X}{Q_c^T X} \times \frac{1}{\eta} \times 100$$

$$Rm(X) = \frac{E^{Cpc(X)}}{E^{Cpm(X)}} \times \frac{Qm}{Qc} \times \frac{Vc}{Vm} \times \frac{1}{\eta} \times 100$$

$$Rm(X) = \frac{E^{Cpc(X)}}{E^{Cpm(X)}} \times \frac{E^{Cpm(COX1)}}{E^{Cpc(COX1)}} \times 100$$

Mitochondrial spatial distribution can be corrected considering contamination rate, which is the mitochondrial localization rate of RNA without connection to mitochondria (e.g., *ACT1*, *RPL10*).

Correction of contaminations with nonmitochondrial fractions. Mitochondrial fraction is always contaminated with other cellular fractions. One can take into account these contaminations if one assumes that some mRNAs are not connected with mitochondria biogenesis, like *ACT1* or *RPL10*. Note that other mRNAs may be considered more pertinent to assess contamination level.

Since cellular RNA arises from cytoplasm and mitochondria contribution and RNA purified in mitochondrial fraction involves specific RNA interaction and contamination from cytoplasm,

$$Q_c^T X = {}^c Q_m^T X + Q_{Cy}^T X$$

$$Q_c^T X = {}^c Q_m^T X + Rc \times Q_{Cy}^T X$$

This leads to

$${}^c Q_m^T X = \frac{1}{1 - Rc}\left(Q_m^T X - Rc \times Q_c^T X\right)$$

Finally, the corrected mitochondrial spatial distribution can be expressed as

$${}^c Rm(X) = \frac{1}{1 - Rc}\left(Rm(X) - Rc\right)$$

Typical values for mitochondrial spatial distribution before and after correction are presented in **Fig. 1** for *ACT1*, *ATP2*, *ATP3*, *COX4,* and *COX6* transcripts (spatial distribution of *ACT1* is considered to represent the contamination rate).

2. *Microarray data analysis and normalization*. Basic analysis and normalization of data can be conducted using Excel software. A dedicated database can take in charge data management and integrates more sophisticated tools for data analysis (statistical analysis of microarray ***(15)*** or dedicated R packages [http://www.bioconductor.org/], for instance).

   The genomewide comparison of mitochondrially associated RNA vs total RNA requires analysis methods different from standard genomewide gene expression analyses. In this case, we expect a skewed distribution with a significant number of mitochondrially associated RNAs "getting out" of the distribution of total RNA on one side (**Fig. 2**). To solve this problem, several methods are available ***(16)***. The most widely used, which we describe here, is the median percentile rank method, which associates to each mRNA a value between 0 and 100, depending on its position in the ratio distribution, thus reflecting the reproducibility of its enrichment among replicate experiments.

   The first steps of data processing are similar to standard global gene expression analysis. For the spots that have been flagged as "good" during the image analysis, we keep from the result file (*.gpr) the median of the foreground (**F**) and of the local background (**B**) for channel Cy5 (635 nm) and Cy3 (532 nm). The Cy5/Cy3 fluorescence ratio (Rf) is then equal to (F635-B635)/(F532-B532). The mRNA are then sorted according to their Rf. The median percentile ranks (mpR) for each mRNA are calculated using the percentile rank function in Excel. The percentile rank of a mRNA with Rf = X is simply the percentage of mRNA with an Rf that is less than X. We multiply this value (ranging from 0 to 1 in Excel) by 100 to scale the value between 0 and 100 for better convenience. The spots that have been flagged as bad or absent at the image analysis step are given an mpR value of NA.

   What we called the MLR ***(6)*** is the median of the mpR of each mRNA among six microarray experiments. A high MLR thus reflects a reproducible enrichment of

the corresponding RNA in the mitochondrially bound polysomes compared with its abundance in total RNAs.

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