Biolistic transformation of *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* mitochondria

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Running title
Mitochondrial transformation

Abstract

Chlamydomonas reinhardtii and *Saccharomyces cerevisiae* are currently the two microorganisms in which genetic transformation of mitochondria is routinely made. The generation of a large variety of defined alterations as well as the insertion of ectopic genes in the mitochondrial genome (mtDNA) are possible, especially in yeast. Biolistic transformation of mitochondria is achieved through the bombardment of microprojectiles coated with DNA, which can be incorporated into mtDNA thanks to the highly efficient homologous recombination machinery present in *S. cerevisiae* and *C. reinhardtii* organelles. Despite a low frequency of transformation, the isolation of transformants in yeast is relatively quick and easy since several natural or artificial selectable markers are available, while the selection in *C. reinhardtii* remains long and awaits for new markers. Here we describe the materials and technics used to perform biolistic transformation, in order to mutagenize endogenous mitochondrial genes or insert novel markers into mtDNA. Although alternative strategies to edit mtDNA are being set up, so far insertion of ectopic genes relies on the biolistic transformation technics.

Key Words: mitochondrial DNA; genetic transformation; biolistic technics; homologous recombination; mutagenesis and ectopic gene insertion; *Chlamydomonas reinhardtii*; *Saccharomyces cerevisiae*.

1 Introduction

Transformation of the mitochondrial genome can be achieved in two unicellular microorganisms: originally the yeast *Saccharomyces cerevisiae* and later on the green microalga *Chlamydomonas reinhardtii* [1].

In both systems, donor DNA is delivered into mitochondria by microprojectile bombardment (biolistic transformation) and the DNA is inserted into the recipient mitochondrial genome by homologous recombination. The use of a biolistic particle delivery system where thousands of microprojectiles are coated with multiples copies of the donor DNA is an efficient way to target the multicopy mitochondrial genomes since it increases the chances to deliver enough copies of the donor DNA to invade the endogenous polyploid genome. In addition, the use of the adequate size of the microparticles and rupture disk determining the power of the helium shockwave allows the particles to cross both the cell and mitochondrial membranes, as discussed in [2].

In other organisms, mitochondrial transformation has been unsuccessful, but DNA editing of the mitochondrial genome is becoming feasible in both animals [3] and plants [4], using sequence-specific nucleases targeted to mitochondria. However so far insertion of novel genes into the mitochondrial genome is still only possible through biolistic transformation, *i.e.* in *S. cerevisiae* and *C. reinhardtii*, and should potentially be achievable in *Candida glabrata* where biolistic transformation has also been reported [5].

1.1 Chlamydomonas mitochondrial transformation

The first demonstration of mitochondrial transformation in Chlamydomonas has been published in 1993 [6]. The authors used a biolistic delivery system to transform a mutant called *dum1*, that harbors a 1.5 kb terminal deletion of the linear 15.8 kb mitochondrial genome comprising the telomere (500 bp) and most of the *cob* gene (1 kb) encoding the apocytochrome *b* (Figure 1). In addition to this deleted genome, the mutant also contains dimers which result from head-to-head fusions between deleted monomers [6, 7]. The *dum1* mutant is unable to grow in the dark in the presence of acetate because of the loss of the activity of complex III and is a non-reverting mutant, which after selection in the dark allows the recovery of rare respiratory competent cells with intact mtDNA.

The next step in the set-up of mitochondrial transformation was reached in 2005 when Yamasaki and coauthors [8] reported that the same deletion mutant could be transformed using plasmids containing fragments of mtDNA as well as PCR products, again using a biolistic equipment. However, the transformation rates were still low (Table 1). In 2006, we published an optimized protocol for mitochondrial transformation using both PCR fragments and plasmids containing mitochondrial genome fragments of various sizes, using the same strategy of selection in the dark and another deletion mutant as recipient strain (*dum11*) which is essentially the same as *dum1* except that the deletion in the *cob* gene is shorter (0.7 kb) [9] (Table 1, Figure 1).

Table 1: Experimental conditions for mitochondrial transformation in *C. reinhardtii*

Strain	Beads	Exogenous DNA	Transformation rate[1]	References
dum1	Tungsten beads (~1 μM)	Partially purified *C. reinhardtii* mitochondrial genome (0.8 μg per bombardment)	[2]3.5-12.5	[6]
dum1, *dum14*, *dum16*	Gold beads (600 + 100 nm)[3]	Plasmids containing from 1.8 to 5.0 kb mtDNA fragments (5 μg per bombardment)	[2,4]5.5	[8]
		3.8 kb PCR product[5] (5 mg per bombardment)	[2]1.5	
dum11	Tungsten beads (< 1 μM)	Plasmids containing 1.8 to 6.6 kb mtDNA fragments (3 μg per bombardment)	~ 100 – 220[6] (10 – 22[2])	[9]
		PCR product (1.5 μg per bombardment)	~ 133 – 266[7] (13 – 26[2])	

[1] Transformation rates based on data found in the respective papers
[2] Transformation rates expressed in number of transformants per 10^7 cells
[3] Several combinations of gold particles of different sizes have been tested and this combination gave the best results
[4] Best transformation rate for undigested plasmid containing a 5.0 kb mtDNA fragment (left telomere-*cob*-Cterm*nd4*/Nterm*nd5*-*cox1*/right telomere) using *dum1* as recipient strain
[5] PCR product from the left telomere to *nd4*
[6] Best transformation rate for linearized plasmid containing a 6.6 kb mtDNA fragment (left telomere to *cox1*, see Figure 1) using *dum11* as recipient strain, expressed in number of transformants/μg DNA
[7] Best transformation rate for a 6.6 kb mtDNA PCR fragment (left telomere to *cox1*, see Figure 1) using *dum11*, expressed in number of transformants/μg DNA

Mutations in genes encoding subunits of respiratory complex I and a mutation conferring resistance to myxothiazol in *cob* could be introduced. At last, a mutant with intact telomeres at both sides of the genome could be transformed. Afterwards, another recipient strain with a longer deletion extending until the *nd4* gene (Figure 1) was used to force the introduction of mutations in this gene [10, 11], although the transformation rates were much lower (Table 1). In general, most of the transformants were homoplasmic for the mitochondrial genome although heteroplasmic ones could also be recovered, that ultimately could reach the homoplasmic state after subcloning [11].

Finally, two reporter genes, *gfp* and *ble* conferring resistance to zeomycin, have since then been successfully inserted between the left telomere and *cob* using the *dum1* strain as recipient [12, 13]. Although these results open the way to ectopic gene insertion, there was no follow up.

1.2 Yeast mitochondrial transformation

S. cerevisiae mtDNA, called the *rho* factor, has proved to be particularly amenable to manipulation, for several reasons. First *S. cerevisiae* can be fully devoid of mtDNA (*rho*^{0}) but can also tolerate and maintain *rho-* genomes, that contain large deletions. Second, there does not seem to be any clear requirement for a specific replication origin in mitochondrial *rho-* sequences, thus allowing even foreign DNA to be maintained. In *rho-*, the remaining sequences are reiterated so that the *rho-* cells contain the same amount of mtDNA as wild-type cells. Third, *rho-* genomes can be expressed *in trans* and are able to freely undergo homologous recombination with mtDNA sequences in crosses. These properties have proved to be invaluable to set up mitochondrial transformation in *S. cerevisiae* in order to manipulate the mitochondrial genome.

The first proof of concept of mitochondrial transformation has been obtained using a particle gun to bombard sequences of the wild-type *COX1* gene into a strain with a full mtDNA (so-called *rho+*), harboring a non-reverting deletion in the mitochondrial *COX1* (*oxi3*) gene. A fully wild-type strain with normal respiratory function was restored [14].

The same year, a similar biolistic transformation of a *rho*^{0} strain (*i.e*. devoid of mtDNA) with a plasmid containing the mitochondrial *COX2* (*oxi1*) was achieved, and shown to rapidly form what was called a synthetic *rho-* genome. This artificial *rho-* mtDNA was carrying the *COX2* gene and could restore respiratory function upon cross with a *rho+* strain harboring a point mutation or deletion in *COX2*.

These two founding experiments opened the way to the manipulation of the yeast mitochondrial genome virtually at will, and today, 33 years later, these two strategies are still used to mutagenize or insert new genes into mtDNA. Direct transformation into a *rho+* strain requires that a selection can be applied to identify transformation and recombination events. To contrary, transformation in two steps does not depend on a selection. First a synthetic *rho-* is obtained after transformation of a *rho*^{0} strain, then the modified or ectopic sequences are inserted into an otherwise *rho+* genome by homologous recombination, through crosses (generating first a diploid) or cytoduction (generating directly a haploid strain without mixing the nuclear backgrounds).

Although a recent publication reported the use of the Crispr Cas9 system to mutagenize yeast mtDNA [16], the only way so far to modify the genome arrangement or introduce ectopic genes in mtDNA depends on biolistic transformation. A maybe not exhaustive overview of the ectopic genes inserted into *S. cerevisiae* mtDNA is provided in Table 2. Amongst these various reports, the two main fundamental breakthroughs were the

engineering of a site upstream of *COX2* for insertions of ectopic genes while preserving respiratory function [17], and the recoding of a mitochondrially-expressed *ARG8* auxotrophy gene [18], that has been the most widely used mitochondrial reporter, inserted at many sites in the mtDNA and used for a wide variety of studies (Table 2). However, since synthetic genes can nowadays be ordered easily, many novel genes and locations of the genome are open to the creativity of researchers working on mitochondrial functions.

Table 2: Example of relocation of ectopic genes or tag within mtDNA using biolistic mitochondrial transformation

Ectopic sequences	*Loci* in mtDNA	Specific features/remarks	Purpose	First publication	
				Year	**Reference**
URA3	Plasmid with *COX2*	Wild-type auxotrophy genes in universal code; expressed only upon DNA escape into the nucleus	First proof of mtDNA escape	**1990**	[19]
TRP1	5' of *COX2*		Selection of mutations modulating mtDNA escape	**1993**	[17]
Arg8^m	Initially *COX3*. Then *CYTb*, *COX1*, *COX2*, *COX3*, *ATP6*, *ATP8*, *VAR1* in other studies	Recoded gene restoring arginine prototrophy in nuclear *Δarg8* strains; mutant version also available	- Gene replacement, introduction of mutations - Reporter gene for gene expression - Study of translation/assembly feed back loop - Analysis of genetic instability - Cargo for translocation across membrane - Positive selection for mtDNA	**1996**	[18]
GFP^m	*COX3*	Green Fluorescent protein	Visible reporter gene – Fluorescence was low	**2001**	[20]
3HA	*COX2*	Fusion at the C-terminus of Cox2	Mitochondrial gene tagging	**2002**	[21]
Barstar	5' of *COX2*	Inhibition of the toxic effect of the Barnase RNAse	Novel selection system	**2003**	[22]
Rip1^m	*COX1*	Used *S. douglasii* 3'UTR sequences to improve the construct stability	Mitochondrial relocation of a nuclear-encoded respiratory complex III subunit	**2003**	[23]
FUM^m	5' of *COX2*	TCA cycle enzyme	Restricting production of fumarase exclusively within mitochondria	**2010**	[24]
4,5S RNA	5' of *COX2*	RNA component of *E. coli* SRP	Expression of RNA part of the SRP particle	**2013**	[25]
SfGFP^m	5' of *COX2*	Super-folder GFP variant	Monitoring mitochondrial translation	**2018**	[26]

Below we will describe the materials needed, protocols to follow and useful tips to achieve efficient mitochondrial transformation using the biolistic method. Many steps are common between *C. reinhardtii* and *S. cerevisiae*, however there are some specificities; in this case, when numbering some steps which are organism-specific, we will precise "*Cr*" for Chlamydomonas or "Sc" for budding yeast.

2 Materials

2.1 Strains

1. *Cr*. Deletion mutant are typically used, such as *dum1*, *dum11*, *dum14*, *dum16* and *dum22* (see Note 1).
2. *Sc*. The *rho*^{0} or *rho+* recipient strain used are preferably *kar1-1* (to allow subsequent cytoduction) and of DBY947 genetic background (For example DFS160 [18], see Note 2).

2.2 Media and cell preparation

1. *Cr*. Liquid and solid TAP (Tris-Acetate-Phosphate) medium: 2.42‰ (w/v) Tris buffered at pH 6.8-7.0 with 1‰ (v/v) glacial acetic acid, 0,108‰ (w/v) K_2HPO_4 and 0.056‰ (w/v) KH_2PO_4, supplemented with a complex set of oligo-elements [27]. Acetate is used as a reduced organic carbon source to sustain growth in the dark (see Note 3).
2. *Cr*: Solid TAP plates: as liquid TAP but with 1.5% (w/v) agar, 1 cm thick and freshly made (1-2 days before the bombardment).
3. *Sc*. YPR: 1% (w/v) yeast extract, 2% (w/v) Bacto™ peptone, 2% (w/v) raffinose, 0.1% (w/v) glucose, 40 µg/mL adenine (see Note 4).
4. *Sc*. YPD: 1% (w/v) yeast extract, 2% (w/v) Bacto™ peptone, 2% (w/v) glucose, 40 µg/mL adenine.
5. *Sc*. SD-sorbitol: 0.67% (w/v) yeast nitrogen base, 5% (w/v) glucose, 1 M sorbitol, 100 µg/mL adenine, 3% (w/v) agar, other supplements as required 40 µg/µL except for the selection marker. Pour a few days before the bombardment and dry moderately.
6. *Sc*. YPEG-Sorbitol: 1% (w/v) yeast extract, 2% (w/v) Bacto™ peptone, 3% (w/v) glycerol, 3% (w/v) ethanol, 0.1% (w/v) glucose, 1M sorbitol; 3% (w/v) agar (see Note 5).
7. Sterile glass tubes and flasks and shaker at 28 to 30°C.
8. *Cr*. White light neon/LED tubes at 75 µmol photons. $m^{-2}.s^{-1}$ in a cultivation chamber at 23-25°C.
9. Room temperature centrifuge with sterilized bottles or sterile plastic 50 ml tubes.

2.3 Preparation of microprojectiles and DNA precipitation

1. Room temperature and refrigerated centrifuge to spin 1.5 ml tubes at 16000 g.
2. Tungsten powder <1 µm 99.95% (metals basis; Alfa Aesar/Johnson Matthey, item 44210, CAS 7440-33-7; see Note 6).
3. 70% (v/v with sterile water) or 100% Ethanol, room temperature.
4. Sterile water.
5. Glycerol: 50% (w/v), sterilized by autoclaving, −20°C.
6. DNA for mitochondrial transformation of Chlamydomonas or yeast (Note 7).
7. *Sc*. Required for yeast *rho*^{0} strains and optional for *rho+* strains: Qiagen-based midi- or maxipreps (>2 µg/µL) of a plasmidic DNA for nuclear transformation, carrying a nuclear

selection marker and a nuclear replication origin; we often use the vector YEp351 [28] or pRS315 [29] for the *LEU2* marker.
8. Spermidine free base (Sigma): 1 M in sterile water.
9. Calcium chloride: 2.5 M in water, kept at −20°C (Note 8), filter-sterilized.
10. Ethanol 100%, freezer stock (Note 9).

2.4 Bombardment
1. Sterile macrocarrier holders #1652322 (Note 10, 11).
2. Macrocarriers (Bio-Rad #1652335).
3. Rupture disks: 1100 psi (Bio-Rad #1652329; see Note 12).
4 *Cr*. Stopping screens sterilized in a Pasteur oven (Bio-Rad #1652336).
5. 70% ethanol for chamber sterilization
6. PDS-1000/He biolistic gun (Bio-Rad #1652257) with helium bottle, vacuum pump and torque wrench.
7. Metal forceps

2.5 Identification of mitochondrial transformants
1. *Cr*. Proteinase K (20 mg/mL in water), stored at -20 °C.
2. *Cr*. Thermocycler and PCR consumables
3. *Sc*. *rho+ mit−* tester strain carrying a mitochondrial mutation in the region covered by the DNA for mitochondrial transformation and of mating type opposite to the recipient *rho*^{0} strain, for example of strain currently used see [30] (Note 13).
4. SD plates: 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, 2% (w/v) agar; supplements as required at 40 µg/µL.
5. YPD plates: like liquid YPD but with 2% (w/v) agar.
6. YPEG plates: 1% (w/v) yeast extract, 2% (w/v) Bacto peptone, 3% (w/v) glycerol, 3% ethanol, 2% agar (Note 14).

2.6 Mating and isolation of yeast recombinant cytoductants
For mating and isolation of recombinant cytoductants, use a recipient wild-type *rho+* strain or a *mit− rho+* strain. To recover haploid recombinants, this strain must carry the *kar1-1* mutation unless the *rho0* strain used for the transformation (Subheading 2.1., item 2) is already *kar1-1*.

3 Methods

3.1 Overview of the transformation procedure
The device used for the microprojectile bombardment is the PDS-1000/He System, available from Bio-Rad, Inc. A detailed protocol is available on line (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1652249.pdf). The system uses a high helium burst in an evacuated chamber to break a rupture disk which propels at optimal velocity a macrocarrier disk, loaded with DNA-coated microscopic metal particles, towards target cells spread on a Petri plate. Upon bombardment, the DNA introduced into the cells is taken up by the nucleus, and to a lesser extent by the mitochondria.

3.2 Cell culture

1. *Cr.* Cultivate the recipient strain for 2-3 days in liquid TAP medium under continuous light (50-70 µmol photons. $m^{-2}.s^{-1}$) and mild agitation to reach mid-exponential phase (cell density of 2-5 10^6 cells/mL). Typically, 300 mL of culture give between 6 and 15 plates at 10^8 cells/plate.
2. *Cr*. Harvest the cells (2700 g, 10 min, 25ºC) in sterilized bottles, resuspend the pellet in sterile TAP at 5 x 10^8 cells/mL.
3. *Cr*. Spread 200 µL on freshly prepared plates. Let the plates dry moderately in the laminar hood.
4. *Sc*. Inoculate a colony of the cell to bombard in a few ml of liquid YPR and grow for 1 night to 2 days at 30°C with agitation.
5. *Sc.* Inoculate a flask containing a larger YPR volume at 1/100 and grow for 2 to 3 days at 30°C under agitation (Note 15). A 30 ml to 50 mL culture will be enough for 6 bombardments.
6. *Sc*. Harvest cells (3000 rpm, 5 minutes, room temperature) in sterile 50 mL tubes and resuspend in sterile YPD to reach a cell density of 1-5.10^9 cells/mL before spreading on plates. Typically, 30 mL of YPR culture resuspended with 600 µl of YPD are spread onto 6 plates (Note 16) of appropriate Sorbitol medium (SD-Sorbitol with supplements except for the nuclear transformation marker, or YPEG-Sorbitol), between 1 and 3 hours before the bombardment.

3.3 Particle sterilization

1. Weigh 10 to 50 mg of tungsten particles (1mg = 1 shot) in a 1.5 mL microcentrifuge tube.
2. Add 1.5 mL of 70% ethanol (100% ethanol for gold particles), vortex and incubate 10 min at room temperature.
3. Spin down the particles for 15 min at 13000 rpm at room temperature and carefully remove the supernatant.
4. Add 1.5 mL of sterile water, vortex and repeat step 3 immediately.
5. Resuspend at 60 mg/mL in frozen 50% glycerol and keep on ice until needed (Note 17).

3.4 DNA coating

The protocol given below is intended for 6 bombardments (Note 18). Precipitation is conducted on ice, with ice-cold or freezer-stocks of reagents, and the rotor of the centrifuge should be pre-cooled at 2-4°C and used at full speed (e.g. 16000 g). Vortex each time a reagent is added.
1. *Cr*. Prepare a tube with 20 µg of linearized plasmid (1-2µg/µL) or 10 µg of PCR product (1-2 µg/µL) (Note 18)
2. Sc. Prepare a tube with 5 µg of plasmid with the nuclear selection marker (*e.g.* YEp351) and 15 to 30 µg of plasmid or PCR DNA for mitochondrial transformation (Note 19). Omit the first plasmid if bombarding cells plated on YPEG-Sorbitol.
3. Add 100 µL of tungsten particles (Note 20) to the DNA and mix, precipitate the DNA onto the particles by adding of 4 µL of 1 M spermidine and 100 µL of freezer-stored 2.5 M $CaCl_2$, vortexing immediately after each addition. Incubate on ice 10 to 15 min and vortex occasionally (every 2-3 min).
4. Spin down 30s in the pre-cooled centrifuge; discard the supernatant and add 200 µL of freezer-stored 100 % ethanol. The aggregates of particles on the side of the tube should be scraped and broken using the pipette tip (Note 21). Repeat this step at least once and possibly more until the particles resuspend easily.
5. Spin down the tube briefly in the pre-cooled centrifuge; discard the supernatant, taking extreme care not to pipette tungsten particles, and add 60 µL of 100% ethanol. The

suspension is distributed evenly on 6 macrocarriers (Note 22) placed in their holders and the ethanol is evaporated under the hood.

3.5 Bombardment *(supplementary video)*

1. Wash the chamber and all removable parts with 70% ethanol before use (Note 23). Carefully wipe dry all parts since the remaining ethanol will prevent building the strongest possible vacuum.
2. Open the helium gas connection and switch on the vacuum pump.
3. Insert the rupture disk (1100 psi = 7584 kPa) in the retaining cap using sterile forceps and tighten the assembly using the torque wrench (Note 24).
4. *Cr*. For Chlamydomonas transformation only, insert a stopping screen on the macrocarrier assembly system (Note 25).
5. Take the macrocarrier loaded in its holder and load it onto the assembly system so that the face with DNA looks down.
6. Remove the lid or the 100 mm Petri dish with the lawn of cells and place at 7 cm (Chlamydomonas, level 3) or 5 cm (yeast, level 2) from the macrocarrier assembly system (Note 26).
7. Close the door with the latch
8. Press the "vac" button to raise the vacuum and reach a partial vacuum corresponding to 29-29.5 inches Hg (736.6 mm Hg) on the PDS1000-He gage (the higher the better, Note 27).
9. Block the chamber evacuation by quickly pressing on the "hold" button.
10. Press the "fire" button. The PDS1000-He gage pressure gage will raise until the rupture disk bursts open noisily; release the "fire" button immediately.
11. Ventilate the chamber with the "vent" button. If applicable eliminate large macrocarrier fragments laying on the Petri plate with a sterile forceps.
12. *Cr*. Incubate Chlamydomonas plates for one day under continuous light (50-70 µmol photons. $m^{-2}.s^{-1}$) at 23-25 °C, transfer in the dark and incubate 4-8 weeks for selection of respiratory competency.
13. *Sc*. Incubate yeast plates at 28 to 30°C until transformants appear (usually 3 to 5 days).

3.6 Identification of Chlamydomonas transformants

1. Design primers for amplification of a PCR product allowing the identification of the recombinant clones.
2. Check the plates after 4-6 weeks under a stereoscopic microscope to detect microcolonies.
3. After 8 weeks, transfer the plates to dim light (5 µmol photons. $m^{-2}.s^{-1}$) for one week. Transformants are usually found at the periphery of the plates while the control plates are empty.
4. Transfer the colonies to fresh TAP plates.
5. Prepare whole cell extracts (adapted from Randolph et al 1993): transfer 0.5 cm^2 of patches of Chlamydomonas into a microcentrifuge tube and add 35 mL of H_2O, 5 mL of PCR buffer 5X, and 10 mL of proteinase K (20 mg/mL in H_2O).
6. Incubate 1 h at 58°C and 1 h at 95 °C.
7. Spin down briefly with a benchtop microfuge and store the crude extracts at 4°C.
8. Use 1/10 (v/v) of the crude extract for PCR experiments.
9. Sequence the PCR fragment if needed.

3.7 Identification of yeast transformants

After bombardment of a rho^0 recipient strain (Figure 2)

1. Three days after the bombardment, inoculate an appropriate *rho+* mutant (*mit*−) tester strain in liquid YPD to set up a fresh culture.
2. When transformants are grown, prepare plates of SD medium with appropriate supplements and spread a lawn of tester strain on YPD plates.
3. Replica plate the transformants first onto the minimal SD medium, to keep the transformants, and then onto the tester strain lawn to cross the transformants. Mark each plate very precisely to facilitate step 6.
4. Incubate 2 days at 30°C to allow mating and recombination.
5. Replica-plate the YPD cross plate to YPEG medium (or another appropriate medium if scoring another phenotype), in order to detect respiring diploids (see Note 28). Incubate for 2 to 3 days at 30°C (see Note 29).
6. Compare the position of the respiring diploids and of the original transformants to pick, off the bombarded plate (or its direct replica), colonies that correspond to the position of respiring recombinants. Favor positions where transformants are not too dense.
7. Streak the transformants on YPD (on which they will lose the plasmid carrying the nuclear marker) and incubate at 30°C to get single colonies. Repeat the cross and marker rescue analysis with the tester strain as in steps 3 to 6. Usually these cycles of subcloning and retesting must be done three times before obtaining pure stable synthetic rho− clones.

After bombardment of a rho+ recipient strain

8. After a bombardment of cells plated directly on YPEG-Sorbitol medium, only the few colonies that underwent mitochondrial transformation restoring respiratory function will grow.
9. After a bombardment of cells plated on SD-Sorbitol medium to first select nuclear transformants, replica-plate the nuclear transformation directly onto glycerol medium (YPEG or N3) (Figure 3).
10. Homologous recombination will be achieved upon growth on glycerol. After streaking cells on YPD and replica-plating on YPEG, a maximum of 1 to 5% of cells should be unable to grow on glycerol.

3.8 Mating and isolation of recombinant cytoductants starting from synthetic rho- clones

1. Pick a single colony of the synthetic *rho-* and recipient *rho+* strains (see Note 30) and inoculate 5 ml liquid YPD culture overnight. At least one of these two strains (usually the synthetic *rho*−) must carry the *kar1-1* mutation that delays karyogamy, thus allowing mtDNA exchange to occur before nuclear fusion.
2. Mix 0.5 mL of each parent (alternatively 1 mL of synthetic *rho*− and 0.2 mL wildtype *rho+*; see Note 31) in a microfuge tube, spin, remove the supernatant, resuspend in residual liquid, and spread the mixture as a lawn onto a YPD plate.
3. Incubate at 30°C for 4–5 h (typically 4h and 30 min). Check zygote formation under the microscope, you should observe the formation of schmoo projections. Scrape the mating cells from the plate (we use a toothpick) and inoculate a fresh 5 mL culture of liquid YPD medium. Incubate at 30°C with agitation for a few hours to overnight.
4. Make a few serial dilutions of the culture and plate them to obtain single colonies on the appropriate minimal medium, *e.g.* to select for the recipient nuclear genotype and against the donor nuclear genotype, if possible. Alternatively, plate on selective medium if

recombination restores *e.g.* growth on glycerol or without arginine, or on YPD medium if no selection is available. Densities of 50–200 colonies per plate should be obtained.
5. Replica-plate these colonies to medium that reveals the altered phenotype of the recipient strain due to integration of the mutant donor sequences into its mtDNA. For example, print to YPEG to identify clones that have become respiratory deficiency upon integration of a new mutation.
6. To eliminate cytoductants that acquired only the synthetic *rho*− mtDNA by cytoduction, candidate clones can be crossed to other tester strains, like a known *rho*- clone that contains the region you are seeking to mutate. Expected *rho+* recombinant cytoductants will produce respiring diploids upon mating to this known *rho*− strain.

4 Notes

1. The *dum1*, *dum11*, *dum14* and *dum16* mutants present a deletion including the left telomere and part or the entire *cob* gene [7, 31], the *dum22* mutant presents a deletion extending until the *nd4* gene [11] (Figure 1). The *dum19 dum25* mutant is not a deletion mutant and possesses a frameshift deletion in *cox1* (*dum19* mutation) and an in-frame 3 bp deletion in *nd1* (*dum25* mutation) [32]. *dum1* (CC-2255), *dum11* (CC-4098) and *dum22* (CC-4738) are available at 'chlamycollection.org'. *dum14*, *dum16* and *dum19 dum25* are available at the Remacle's lab.

2. The genetic background of the strain is a very important factor for the biolistic transformation efficiency [33]. Mostly we use cells from the S288c background, especially strains derived from DBY947 [34], such as DFS160, a strain harboring an *arg8* deletion which is at once very convenient for bombardment of constructs with the *ARG8*^{m} reporter [18]. Strains derived from W303 [35], (ATCC 200060) give lower but satisfactory efficiencies whereas strains in the D273-10B (ATCC 24657) background are very difficult to transform. In addition bombarding a *rho+* strain is 10 to 20 less efficient than bombarding a *rho*^{0} (compare Figures 2 and 3).

3. TAP medium (1 ‰ (v/v) glacial acetic acid, corresponding to 17.3 mM sodium acetate) is the most common used medium but initial experiments have been made with HSHA high-salt high-acetate medium which contains 29.4 mM sodium acetate [27]. Some authors add ampillicin 50 mg/mL in the dark to avoid contaminations. We have not tried and found no excessive contaminations provided that the chamber where the bombardment takes place is sterilized just before use.

4. The 0.1% glucose accelerates growth, it is optional. Since raffinose is expensive, galactose can also be used without significant decrease in transformation efficiency. Adenine is added even for Ade+ prototrophs, since it seems to increase the transformation efficiency.

5. YPEG supplemented with 1M sorbitol can be used for direct selection of respiring transformants into *rho+* cells; the 0.1% glucose supplement accelerates growth. Alternatively, *rho+* cells can also be plated on the usual SD-Sorbitol medium for bombardment and directly replicated onto YPEG medium to select respiring transformants (see Figure 3). In addition, when selecting for transformants that are expected to produce the functional Arg8 protein by expression of the *Arg8*^{m} reporter gene, cells can be directly plated on SD-Sorbitol medium lacking arginine.

6. Tungsten powder M10 ~0.7 µm and gold powder 0.6 µm are also available from Bio-Rad (catalog #1652266 or #165–2262, respectively). However we always use Alfa Aesar tungsten powder <1 µm which is inexpensive and works as well as gold particles, and 3 times better than the tungsten powder from Bio-Rad which is the process to be discontinued.

7. In Chlamydomonas, the transforming DNA is preferably a linearized plasmid DNA or a PCR fragment, although super-coiled DNA can also be used, albeit with lower efficiency. In yeast, the DNA used for mitochondrial transformation is preferably a concentrated plasmid DNA, although successful transformation has also been achieved with PCR fragments (minimal size for recombination=28 bp). When plasmids are used, Qiagen plasmid midi or maxi kits (tip-100 or tip-500) give the best results in our hands. The transforming DNA concentration should be at least 2 µg/µL for plasmids.

8. Frozen 2.5M $CaCl_2$ remains liquid, so no need to aliquote.

9. If available use a brand new bottle of absolute ethanol bottle and freeze 15 ml aliquotes.

10. Usually we soak macrocarriers holders for one night in absolute ethanol after each bombardment, then drain and sterilize them in a Pasteur oven.

11. It is possible but very unconvenient to make during the same experiment more shoots than the number of available macrocarrier holders. Thus for routine use, investing in more holders will rapidly be worth the price to optimize the number of strains constructed per biolistic transformation experiment.

12. Rupture disks of 1350 psi (pound per square inch) are also efficient for biolistic transformation of yeast, but in our hands 1100-psi disks rather give better results. If rupture disks are very old (several years), they can still be used, however they tend to be less resistant and will break at lower helium pressure, then 1350 psi might be more efficient than 1100 psi.

13. If possible it is best to use a tester in a background like DBY947 rather than D273-10B, since for tester crosses (subheading 3.7 step 1) the lawn will be less thick and will not mask the print of the transformants, making it easier to identify positive colonies

14. Alternatively we use N3 medium: 1% Yeast Extract, 1% Bacto-Peptone, 2% glycerol, 0.05 M Potassium Phosphate pH= 6.2.

15. In our hands a 3-day culture is optimal for *rho0* or *rho+* cells from the DBY947 background.

16. We prefer spreading the cells with a glass rod rather than beads because plating will be more heterogenous, thus providing zones of optimum cell density for the transformation as well as an irregular pattern of transformants: this provides useful identification marks when comparing the plates to pick the mitochondrial transformants at Subheading 3.7., step 6.

17. Sterilized tungsten particles can be kept frozen in 50% glycerol for several months to several years without loss of transformation efficiency. Discard if the supernatant turns yellowish. We have not tried gold.

18. Precipitations can be upscaled by a factor of 1.5 or even 2, still in the same tube; however keep using a 200 µl tip to resuspend and break the precipitate with ethanol at step 4, even if the ethanol volume is upscaled, because using 1 mL tips will not yield a finely dispersed DNA-particle precipitate. Using siliconized tubes for the precipitation did not make a significant difference for us.

19. Keeping the DNA volume to a minimum optimizes the precipitation.

20. Particles settle extremely rapidly, vigorously vortex the tungsten suspension each time before pipetting.

21. Conduct this step thoroughly to obtain a finely dispersed precipitate that will be crucial to efficiently transform the cells.

22. We usually work under a laminar flow hood, using sterile forceps or the specific Bio-Rad tool to insert the macrocarrier membranes into the holders placed into sterile sterile Petri dishes. Do not prewash the macrocarriers nor desiccate them after coating; the ethanol will quickly evaporate.

23. For a very effective sterilization, the chamber and parts can be soaked with 70% ethanol before starting the cell preparation and DNA precipitation, allowed to sit, and wiped dry just before the bombardment. Using a 70% ethanol spray is convenient. The chamber has to be washed in a similar way after the bombardment and even more thoroughly, to remove any residues of agar medium that would lead to contaminations later on.

24. Contrary to Bio-Rad recommendations, we never soak rupture disks in isopropanol just before use. This reduces the transformation efficiency and is unnecessary to prevent contamination. Be careful not to use two rupture disks stacked together, they won't break at the desired helium pressure. It is easy to notice because in this case the hole for tightening the retaining cap with the torque wrench will not be exactly at the usual place.

25. In yeast we have found that more transformants were obtained when omitting to assemble the stopping screen before the microcarrier. However, in this case it is important to use 3% agar in the sorbitol plates, as advised in Subheading 2, otherwise the flying carrier disk will severely damage the agar surface.

26. Shorter distances result in very high colony densities in the center of the plate and few colonies at the periphery while longer distances decrease the transformation efficiency.

27. Failure to draw the greatest possible vacuum dramatically reduces the transformation efficiency. Teflon sealing tape can be used to reduce air leakage in the connections between the chamber and the pump. Cell viability is not significantly affected by an extended stay under these vacuum conditions.

28. For plates with a large number of transformants, it is useful to replicate the mated cells also on glucose medium selecting for the diploids; these diploid plates may by comparison facilitate the identification of the mitochondrial transformants on the original bombarded plates.

29. Respiratory-proficient colonies will appear earlier if the transformed DNA allows complementation of the *mit*− mutation already *in trans*.

30. If available, it is convenient to use as recipient a *rho+* strain carrying a deletion mutation in the region of interest. This deletion should be large enough to include the change created and small enough to still allow recombination with the synthetic *rho*− sequences.

31. If the synthetic *rho*− donor and the *rho+* recipient strains have similar auxotrophies and thus cannot be easily distinguished, selected or counter-selected on minimal glucose medium, mating mixtures should contain equal numbers of cells of both strains. If some markers allow selection *e.g.* against the synthetic donor, then a fivefold excess of donor cells should be used compared to the recipient strain. In the cytoduction experiment, it might be convenient to recover at once recombined *rho+* cells in both parent backgrounds.

Supplementary material: Bombardment.mp4

Acknowledgments
We are indebted to Claudia Serot for generating the supplementary video showing the bombardment procedure, including filming, choosing the sequences and editing the video clip. N. B. is supported by the Centre National pour la Recherche Scientifique, and C.R. is supported by Fonds National de la Recherche scientifique (FNRS, CDR J.0175.20) and Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO) and FNRS under EOS Project No. 30829584.

Figure legends

Figure 1 : Physical map of the 15.8 kb linear mitochondrial genome (GenBank accession EU306622). Horizontal blue arrows indicate the bidirectional transcription origin. The blue boxes represent the eight protein-coding genes: *cob* (apocytochrome *b* of complex III), *cox1* (subunit 1 of cytochrome *c* oxidase or complex IV), *nd1*, *2*, *4*, *5*, and *6* (subunits of NADH:ubiquinone oxidoreductase or complex I) and *rtl* (reverse transcriptase-like protein). The boxes in two shades of green represent *LSU* (*L1*-*L8*) and *SSU* (*S1*-*S4*) genes encoding the structural ribosomal RNAs fragments. The vertical bars correspond to the 3 tRNA genes with the one-letter code (*W*, *Q*, and *M*). The light blue arrows at each end of the genome represent the inverted terminal repeats.

Figure 2 : Production of a synthetic *rho*- yeast strain by biolistic transformation. The [Leu-] *rho*^{0} strain DFS160 [17] was bombarded with a mixture of a *LEU2* plasmid YEp351 and a plasmid containing the *COX2* gene. The tester strain NB97 carried a ~130nt deletion in *COX2* (*cox2-60*, [30]. Steps are numbered as indicated in the text in the method section.

Figure 3: Example of *rho+* yeast mitochondrial transformants obtained directly after bombardment of a *rho+ mit*- strain. A lawn of the *rho+ cox2-62* strain NB41 [30] that carries a 660 bp deletion in the upstream region of *COX2* was spread onto medium lacking leucine and bombarded with a plasmid containing roughly 3 kb of ectopic sequences inserted upstream of *COX2* in a region contained within the *cox2-62* deletion, as well as a plasmid with the nuclear *LEU2* marker gene (YEp351). [Leu+] transformants obtained after a 5 day-incubation at 30°C were directly replica-plated onto glycerol medium to select mitochondrial transformants, for which respiratory growth is restored.

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