A novel system to monitor mitochondrial translation in yeast

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ABSTRACT The mitochondrial genome is responsible for the production of a handful of polypeptides that are core subunits of the membrane-bound oxidative phosphorylation system. Until now the mechanistic studies of mitochondrial protein synthesis inside cells have been conducted with inhibition of cytoplasmic protein synthesis to reduce the background of nuclear gene expression with the undesired consequence of major disturbances of cellular signaling cascades. Here we have generated a system that allows direct monitoring of mitochondrial translation in unperturbed cells. A recoded gene for superfolder GFP was inserted into the yeast (Saccharomyces cerevisiae) mitochondrial genome and enabled the detection of translation through fluorescence microscopy and flow cytometry in functional mitochondria. This novel tool allows the investigation of the function and regulation of mitochondrial translation during stress signaling, aging and mitochondrial biogenesis.

INTRODUCTION

In Saccharomyces cerevisiae the mitochondrial genome encodes eight proteins of which seven are membrane proteins and core subunits of the oxidative phosphorylation system (OXPHOS) [1]. Respiratory activity, therefore, depends on mitochondrial translation, but the molecular mechanisms, regulation and timing of mitochondrial translation are largely unexplored [2]. A major obstacle when analyzing mitochondrial translation in cells is the need to inhibit cytosolic translation in order to follow incorporation of radiolabeled amino acids into mitochondrial translation products [3]. Such pharmacological inhibition impacts severely on cellular homeostasis and alters signaling cascades. For example, inhibition of cytosolic translation using cycloheximide leads to an increase in free amino acid levels, which in turn activates TOR signaling [4-6] and thus affects cell growth, nutrient sensing and life span [7-9]. Additionally, cycloheximide treatment alters protein degradation [10] and acutely impacts on mitochondrial translation within very short time frames [11]. To directly monitor mitochondrial protein synthesis without interfering with cellular protein homeostasis new experimental tools are needed.

Employing biolistic transformation we have integrated a gene encoding superfolder GFP into the mitochondrial genome. This reporter is compatible with mitochondrial translation, respiratory activity, therefore, depends on mitochondrial translation, but the molecular mechanisms, regulation and timing of mitochondrial translation are largely unexplored [2]. A major obstacle when analyzing mitochondrial translation in cells is the need to inhibit cytosolic translation in order to follow incorporation of radiolabeled amino acids into mitochondrial translation products [3]. Such pharmacological inhibition impacts severely on cellular homeostasis and alters signaling cascades. For example, inhibition of cytosolic translation using cycloheximide leads to an increase in free amino acid levels, which in turn activates TOR signaling [4-6] and thus affects cell growth, nutrient sensing and life span [7-9]. Additionally, cycloheximide treatment alters protein degradation [10] and acutely impacts on mitochondrial translation within very short time frames [11]. To directly monitor mitochondrial protein synthesis without interfering with cellular protein homeostasis new experimental tools are needed.

Using biolistic transformation we have integrated a gene encoding superfolder GFP into the mitochondrial genome. This reporter is compatible with mitochondrial translation, respiratory function and enables the direct detection of translation through fluorescence microscopy and flow cytometry in functional mitochondria. This novel tool will facilitate future studies on the regulation and timing of mitochondrial translation.

RESULTS AND DISCUSSION

Integration of a superfolder GFP gene into the mitochondrial genome

In a pioneering previous study, a gene encoding fluorescence-enhanced GFP was inserted into the mitochondrial genome to replace the open reading frame of COX3 [12]. The resulting strain was respiration deficient and expressed only weak fluorescence, which limited its usefulness in fluorescence microscopic and flow cytometric experiments. This limitation could be explained by poor folding of GFP expressed in the context of the mitochondrial

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Abbreviations:
CAP – chloramphenicol, OXPHOS – oxidative phosphorylation, sfGFP – superfolder GFP, UTR – untranslated region.
translational system [13], which is specialized on the production of membrane proteins. To circumvent the folding problem, we employed superfolder GFP (sfGFP), which folds with enhanced kinetics resulting in a more stable protein [14]. To allow translation of the mRNA by mitochondrial ribosomes its coding sequence was flanked by the authentic 5' and 3' untranslated regions (UTR) of COX2 [15-18], coding for the cytochrome oxidase subunit Cox2. To avoid the respiratory deficiency associated with inactivation of mitochondrial genes, we engineered a new mitochondrial genome that coded for sfGFP as an additional ninth open reading frame. The final engineered gene was termed sfGFPm and was cloned into the pPT24+ plasmid (Fig 1A-B), yielding a plasmid (pPT24+-sfGFPm) that contained COX2 5'UTR-sfGFPm-COX2 3'UTR as well as the authentic COX2, including its upstream sequences (Fig 1B). pPT24+-sfGFPm was delivered into a strain that lacks mtDNA (rho0) using biolistic transformation [19]. After selection, the resulting strain that carried pPT24+-sfGFPm was used to cytoduce strain cox2-62, which lacks a functional COX2 gene, to gain respiratory competence by homologous recombination of the two mitochondrial genomes [20]. Finally, we transferred the novel mitochondrial genome into the strain W303 resulting in the strain sfGFPm. Western blot analysis demonstrated that this engineered genome expressed readily detectable levels of sfGFPm (Fig 1C).

Expression of mitochondrially encoded sfGFPm does not disturb mitochondrial function

We assessed the impact of sfGFPm expression on mitochondrial function by monitoring respiratory growth on non-fermentable medium and found that it was similar between wild type and the sfGFPm strain (Fig 2A and 2B). As predicted, steady state levels of respiratory chain subunits were unchanged and showed the expected increase when cells were grown in non-fermentable compared to fermentable medium (Fig 2C). Finally, we checked for respiratory chain assembly into supercomplexes as well as cytochrome oxidase activity and found that both were unchanged compared to wild type (Fig 2D). We therefore conclude that expression of sfGFPm does not alter the assembly of respiratory complexes or the general respiratory competence of the cells.

Expression of sfGFPm followed by flow cytometry

We next explored the possibility to use sfGFPm as an optical readout for mitochondrial translation. First we checked the steady-state levels of sfGFPm by Western blotting during respiration and fermentation to verify that mitochondrially encoded sfGFPm follows the same expression pattern as other mitochondrially encoded proteins. As expected sfGFPm protein levels increased in the presence of galactose and glycerol after 6 and 8 hours in the same way as Cox2 levels (Fig 3A-B). Next we determined GFP fluorescence by flow cytometry under the same conditions and observed an increase in the fluorescent signal when cells were grown for 6, 8 and 10 hours in galactose or glycerol containing medium (Fig 3C-D). To control for mitochondrial biogenesis we used mCherry-tagged Cit1, a nuclear encoded protein that is imported into mitochondria from the cytosol [21]. Similar to sfGFPm, Cit1-mCherry levels also increased when grown in the presence of galactose or glycerol, indicating a general increase in mitochondrial biogenesis induced by galactose and glycerol (Fig 3C-D). In sum, these results confirm that sfGFPm represents a functional reporter for the analysis of mitochondrial translation in vivo without the need to inhibit cytosolic translation. Flow cytometry as quantitative readout opens up many
possibilities to interrogate mitochondrial translation with single cell resolution and allows the combination of sfGFPm with additional fluorophores for a multitude of simultaneous analyses.

**sfGFPm allows detection of changes in mitochondrial gene expression**

We determined the stability of sfGFPm by inhibiting mitochondrial translation using chloramphenicol (CAP). Using immunoblotting coupled with detection by near-infrared fluorescence, we found that sfGFPm levels declined over four hours after treatment with CAP (Fig 4A). Accordingly, flow cytometry confirmed that already two hours after block of mitochondrial translation via CAP, a decrease in GFP intensity was detectable. While this loss of sfGFPm signal became even more evident over time (Fig 4B), the nuclear encoded mitochondrial protein Cit1-mCherry was stable (Fig 4B). To further test the reporter construct, we made use of glucose repression of mitochondrial function [22-24]. When adding glucose to non-fermentable medium, mitochondrial biogenesis as well as translation and OXPHOS assembly are repressed. During glucose repression, we observed a pronounced decline in sfGFPm levels by Western blotting and flow cytometric evaluation. As expected, Cit1-mCherry levels were also decreased by glucose-induced repression of mitochondrial biogenesis (Fig 4C-D). Taken together, both western blot analysis and flow cytometric evaluation of sfGFPm faithfully report the specific inhibition of mitochondrial translation by CAP as well as the general down regulation of mitochondrial biogenesis during glucose repression.

**sfGFPm visualized by fluorescence microscopy**

Finally, we visualized sfGFPm via fluorescence microscopy. Cells grown in non-fermentable medium in exponential phase showed mitochondrial localized GFP fluorescence (Fig 5), as evidenced by co-localization with Cit1-mCherry (Supplemental movie). This opens the possibility to employ sfGFPm not only as a reporter for mitochondrial translation but also to follow mitochondrial movement during cell division, mitophagy, or mitochondrial biogenesis.

In conclusion, we have established a functionally neutral mitochondrially encoded GFP reporter, termed sfGFPm, which is easily monitored via Western blotting, flow cytometry and microscopy. Our reporter allows studying mitochondrial translation in vivo without poising cytoplasmic translation and therefore enables detailed studies of mitochondrial function without perturbing cell physiology. The versatile nature of the new mitochondrial genome described here opens up new venues for the investigation of mitochondrial gene expression by optical methods in respiring cells.

**MATERIALS AND METHODS**

**Generation of sfGFPm**

A gene encoding superfolder GFP (sfGFP) was was recoded to match mitochondrial codon usage, synthesized (Invitrogen, Life technologies, GeneArt) and cloned into the pKM vector using Ndel and Xhol. pKM contains the 5' and 3' UTR of COX2.
between two EcoRI restriction sites. sfGFP was flanked by the 5′ and 3′ UTR of COX2 was then re-cloned from pKM into pPT24* using the EcoRI restriction sites. pPT24*-sfGFP contains a part of authentic mtDNA, the sfGFP under control of the 5′ and 3′ UTR of COX2 and, downstream of sfGFP, the full COX2 gene including its 5′ and 3′ UTR. pPT24*-sfGFP was integrated into the mitochondrial genome via biolistic transformation and homologous recombination. Briefly, the rho0, kar1-1 DFS160 strain was grown in YP medium containing 2% raffinose and spread on SD-Loa plates containing sorbitol (1 M). The nuclear marker plasmid pRS315 (5 µg), as well as the pPT24*-sfGFP (10-15 µg), were precipitated on tungsten particles (100 µl tungsten particle (0.5 µm), 1 M CaCl2, 16 mM spermidine) and spread on flying discs. The particles were bombarded onto a lawn of yeast cells with a particle gun (Bio-rad, USA). After four to five days positive nuclear transformants were collected on a master plate and crossed to the pPT24* strain MOY1360 (Mat a ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1, CIT1-mCherry::TRP1, sfGFPm::cox2, COX2). Cells were grown at 30°C in 1% yeast extract, 2% peptone medium supplemented with 2% dextrose (YPD), 2% galactose (YPGal) or 2% glycerol (YPG) under shaking. When indicated 2 mg/mL chloramphenicol (dissolved in ethanol) or 150 mg/mL cycloheximide (dissolved in water) were added.

SDS-PAGE and Western Blotting

Proteins were extracted by alkaline lysis with 370 mM NaOH, precipitated with 8.33% TCA and pellets were resuspended in reducing sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT) (modified from [29]). Proteins were separated using 16% acrylamide, 0.2% bisacrylamide SDS-PAGE and blotted on nitrocellulose membrane. Western blot signals were quantified using ImageJ. For immunoblot analyses upon treatment with chloramphenicol (CAP) or addition of 2% glucose, cells equivalent of 4 OD600 were harvested at indicated time points. Proteins were extracted by alkaline lysis with 1.8 M NaOH, 7.5% β-mercaptoethanol and precipitated with 27.5% TCA. Pellets were resuspended in 100 µL urea loading buffer (200 mM Tris-HCl pH 6.8, 8 M Urea, 5% SDS, 1 mM EDTA, 0.02% Bromphenolblue, 15 mM DTT) and incubated at 65°C for 10 min. Proteins were separated on 12.5% Tris-
glycine SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour with 1% skim milk in TBST (0.05% Tween) and incubated overnight with antibodies directed against alpha-tubulin (ab184970) and GFP (Roche 11814460001) in TBST (0.2% Tween + 1% skim milk). Membranes were washed four times with TBST (0.05% Tween) and incubated over night with antibodies directed against alpha-tubulin (ab184970) and GFP (Roche 11814460001) in TBST (0.2% Tween + 1% skim milk). Membranes were blocked for 1 hour with 1 % skim milk in TBST (0.05% Tween) and incubated overnight with antibodies directed against alpha-tubulin (ab184970) and GFP (Roche 11814460001) in TBST (0.2% Tween + 1% skim milk).

Native-PAGE and complex IV activity
For native-PAGE, isolated mitochondria (100 µg) were solubilized (50 mM BisTris, 25 mM KCl, 1 mM EDTA, 2 mM aminoheptonic acid, 12% glycerol, 1 mM PMSF, 1x complete, 2% digitinon) for 10 minutes on ice. After a clarifying spin, lysates were separated on a native 3-12% Bis-Tris gel (NativePAGE, ThermoFisher). Supercomplexes were visualized by coomassie staining or complex IV activity assay. Briefly, complex IV activity was visualized by adding 2.5 mM 3,3'-Diaminobenzidine dissolved in 0.05 M phosphate buffer pH 7.4, 1 nM catalase, 1 mg/ml cytochrome c and 240 mM sucrose.

Fluorescence microscopy
Cells were grown at 30°C and live images were taken using a Zeiss LSM 800 Airyscan microscope (Carl Zeiss, Jena, Germany) with a Plan-apochromatic 63X/1.4 numerical aperture oil immersion lens. For confocal excitation of GFP, a 488-nm diode laser was set at 10.0%, and emission was detected between wavelengths 492 and 540 nm. For excitation of mCherry, a 561-nm laser line was used at 2.8%, and emission was detected between 565 and 695 nm. A Z-stack of cells was performed using ZEN blue 2.1 software and maximum intensity projections of the images are shown. Image analysis was done by using ImageJ (National Institutes of Health, Bethesda, MD) and Imaris 8.4 software.

Flow cytometry
1x10^6 cells were harvested via centrifugation. Cells were washed once with water and resuspended in PBS (PBS, 25 mM
potassium phosphate; 0.9% NaCl; adjusted to pH 7.2). For quantification of fluorescence intensities using flow cytometry (BD LSR Fortessa), 30,000 cells were recorded and analyzed with the BD FACSDiva software. A W303a strain carrying no fluorescent tag served as background, and respective fluorescence intensities on either YPD, YPGal or YPGly were subtracted as background.

**Statistics**
Statistical significance was determined using student’s t-test (unpaired, two-tailed, equal variance).

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**SUPPLEMENTAL MATERIAL**
All supplemental data for this article are available online at www.microbialcell.com.

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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