Visualizing Mitochondrial Importability of a Protein Using the Yeast Bi-Genomic Mitochondrial-Split-GFP Strain and an Ordinary Fluorescence Microscope

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Running Title: Visualizing mitochondrial proteins using BiG Mito-Split-GFP

Key words

Mitochondria, localization, dual localized, BiG Mito-Split-GFP, living cells, *Saccharomyces cerevisiae*, epifluorescence microscopy

Abstract

Proving with certainty that a GFP-tagged protein is imported inside mitochondria by visualizing its fluorescence emission with an epifluorescence microscope is currently impossible using regular GFP-tagging. This is particularly true for proteins dual localized in the cytosol and mitochondria, which have been estimated to represent up to one third of the established mitoproteomes. These proteins are usually composed of a surpassingly abundant pool of the cytosolic isoform compared to the mitochondrial isoform. As a consequence, when tagged with a regular GFP, the fluorescence emission of the cytosolic isoform will inevitably eclipse that of the mitochondrial one and prevent the detection of the mitochondrial echoform. To overcome this technical limit, we engineered a yeast strain expressing a new type of GFP called Bi-Genomic Mitochondrial-Split-GFP (BiG Mito-Split-GFP). In this strain one moiety of the GFP is encoded by the mitochondrial DNA while the second moiety of the GFP can be tagged to any nuclear-encoded protein (suspected to be dual localized or bona fide mitochondrial). By doing so, only mitochondrial proteins or echoforms of dual localized proteins, regardless of their organismal origin, trigger GFP reconstitution that can be visualized by regular fluorescence microscopy. The strength of the BiG Mito-Split-GFP system is that proof of the mitochondrial localization of a given protein rests on a simple and effortless microscopy observation.

1. Introduction

Fusing green fluorescent protein (GFP) or any other fluorescent protein still remains the fastest approach to visualize, using epifluorescence or confocal microscopy, the localization of a given protein in a given subcellular compartment and notably in mitochondria. However, a growing number of proteins studied in yeast and Human, display a dual cytosolic and mitochondrial localization [1, 2]. It is therefore very difficult, if not impossible, to distinguish the fluorescence of the mitochondrial pool (also called mitochondrial echoform) of such kind of GFP-tagged dual-localized protein from the cytosolic pool (cytosolic echoform) by epi- or confocal microscopy [3]. The fact that, in most cases, the proportion of the cytosolic pool largely exceeds that of the mitochondrial one, dramatically accentuates this difficulty. Confirming the mitochondrial relocation of a small pool of a cytosolic protein usually necessitates obtaining highly pure mitochondria by subcellular fractionation and verifying the purity of the mitochondria using antibodies directed against sub-compartment-specific markers. However, getting ultra-pure mitochondria devoid of cytosolic contaminants is technically almost impossible to achieve since disrupting and isolating compartments tightly bound together in the cell *via* contact sites like the ER-mitochondria ERMES complex [4] or the vacuole-mitochondria vCLAMP [5], is currently very challenging and laborious.

Owing to the constantly increasing number of mitochondrial echoforms in established mitoproteomes, there is a crucial need for a reliable unbiased, cost effective and rapid new tool that could validate the presence of a protein inside mitochondria and that also enables the specific visualization of only the mitochondrial echoform of a dual-localized protein. To this end, we re-engineered the two-fragments self-assembling Split-GFP originally designed by Cabantous and co-workers [6] and engineered a yeast strain harboring a so-called Bi-Genomic Mitochondrial-Split-GFP (BiG Mito-Split-GFP). In this strain, the gene encoding the ß1-10

fragment of the Split-GFP ($GFP_{ß1-10}$) was biolistically integrated [7, 8] into the mitochondrial genome and is thus only translated inside the mitochondrial matrix by mitoribosomes. On the other hand, the second Split-GFP fragment ($GFP_{ß11}$) sequence can be fused to any nuclear-encoded protein that will be translated by the cytosolic translation machinery **Fig. 1**. By doing so, the cytosolic echoform of $GFP_{ß11}$-tagged dual-localized protein will not generate a GFP signal because it will never be in contact with the $GFP_{ß1-10}$ complementing fragment which is synthesized and entrapped inside the mitochondria. On the opposite, upon mitochondrial import, the $GFP_{ß11}$ fragment fused to the mitochondrial echoform will be able to interact with the mitochondrially-produced $GFP_{ß1-10}$ and trigger assembly of a fully functional GFP emitting a fluorescent signal that can be visualized by conventional fluorescence microscopy. To guarantee that any mitochondrial protein will generate a fluorescent signal regardless of its expression level, we did not use a single ß11 to tag the nuclear-encoded gene of interest but concatenated three β11 strands (β11-chaplets or $GFP_{ß11ch}$, [7]) and mutagenized both original Split-GFP fragments in order to increase their stability and self-assembly [7, 9]. The BiG Mito-Split-GFP system not only allows specific and unbiased visualization of discrete pools of mitochondrial echoforms of yeast dual-localized proteins but also constitutes a suitable and reliable tool to test mitochondrial importability of proteins from other organisms like plants and Human [7]. We describe herein (i) how to generate the plasmid expressing the $GFP_{ß11ch}$-tagged gene of interest, (ii) how to transform and grow the BiG Mito-Split-GFP strain, (iii) how to visualize the mitochondrial GFP signal with an ordinary epi-fluorescence microscope and (iv) how to verify expression of the $GFP_{ß11ch}$ and $GFP_{ß1-10}$ fragments using commercially-available antibodies.

2. Materials

2.1. PCR and Isothermal assembly

Description of the buffers and isothermal assembly enzymes used to fuse the $GFP_{ß11ch}$ sequence at the 3'-end of the gene of interest (GOI). The design of the oligonucleotide primers used for the PCR and isothermal assembly of the plasmid are described in **Fig. 2**.

1. Thermal Cycler (C1000 TouchTM Thermal Cycler (BIO-RAD)).
2. 5× isoT master mixture (mix): 3 mL Tris-HCl pH 7.5 1 M, 150 µL $MgCl_2$ 2 M, 600 µL of dNTP mix (10 mM each), 300 µL DTT 1 M, 300 µL NAD^+ 100 mM, 1.5 g of PEG-8000 and sterile ultrapure water up to 6 mL final volume. Store 320 µL aliquots in a -80 °C freezer.
3. 2× reaction master mix: 320 µL 5× isoT master mix, 1.2 µL of T5 exonuclease (New England Biolabs, 10 U/µL), 20 µL Phusion DNA polymerase (ThermoFisher Scientific, 2 U/µL), 8 µL Taq DNA ligase (New England Biolabs, 40 U/µL) and water up to 800 µL. Freeze 11 µL aliquots and keep at -80 °C.
4. Dry Heating block for 1.5 mL microtubes.

2.2. BiG Mito-Split-GFP transformation

1. BiG Mito-Split-GFP Strain (see **Table 1**).
2. Liquid Yeast extract Peptone Dextrose (YPD): Peptone 2 % (w/v), Yeast extract 1 % (w/v), Dextrose 2 % (w/v). Autoclaved at 120 °C, 1.2 bars for 20 min.
3. Bench Top Centrifuge.
4. Dry Heating Block for 1.5 mL microtubes.
5. Salmon sperm DNA (10 mg/mL).
6. Lithium acetate 1 M (no need to adjust pH).
7. Polyethylene glycol (PEG)-4000 50 % (w/v).
8. Water bath.

9. Solid Synthetic Complete Dextrose without tryptophane (SCD-Trp): 0.675 % Yeast Nitrogen Base (without amino acids), 2 % Dextrose, amino acid (-Trp) dropout mix, 2 % agar. Autoclaved at 120 °C, 1.2 bars for 20 min.
10. Glass beads (2.5 – 4.5 mm in diameter).
11. Incubator.

2.3. BiG Mito-Split-GFP growth

1. Liquid Synthetic Complete Dextrose without tryptophane (SCD-Trp): 0.675 % Yeast Nitrogen Base (without amino acids), 2 % Dextrose, amino acid (-Trp) dropout mix. Autoclaved at 120 °C, 1.2 bars for 20 min.
2. Incubator Shaker
3. Liquid Synthetic Complete Galactose without tryptophane (SCGal-Trp): 0.675 % Yeast Nitrogen Base (without amino acids), 2 % Galactose, amino acid (-Trp) dropout mix. Do not autoclave, but rather use vacuum filtration system with 0.2 µm pores.

2.4. Epi-fluorescence Microscopy

1. AXIO Observer d1 (Carl Zeiss) epifluorescence microscope using a 100 × plan apochromatic objective (Carl Zeiss).
2. 76 × 26 mm microscope slide and 22 × 22 mm cover slip.
3. MitoTracker™ Red CMXRos (Thermo Fisher).
4. Liquid SC-Gal: 0.675 % Yeast Nitrogen Base (without amino acids), 2 % Galactose, aminoacid (-Trp) dropout mix. Do not autoclave, but rather use vacuum filtration system with 0.2 µm pores.

2.5. SDS-PAGE electrophoresis, Western Blotting and Immunodetection

1. NaOH 0.185 M.
2. Trichloroacetic acid 100 % (w/v).
3. Laemmli buffer 1 × (see **Note 1**).

4. Mini-PROTEAN® 3 System Glass Plates (BIO-RAD)
5. SDS polyacrylamide gels. The resolving gel is composed of acrylamide:bisacrylamide (30% 37.5:1) supplemented with 0.5 % (v/v) 2,2,2-Trichloroethanol (TCE) (optional, see **Note 2**) diluted in 450 mM Tris-HCl pH 8.8, 0.12 % (w/v) SDS and the acrylamide concentration is adjusted depending on the molecular weight of the proteins to analyze (usually 8- , 10- or 12 % (v/v)). After addition of 0.1 % (w/v) ammonium persulfate (APS) and 0.1 % (v/v) N,N,N',N Tetramethylethylenediamine (TEMED), immediately pour the solution between two 10 × 8 cm Mini-PROTEAN® Spacer Plates from BIO-RAD with integrated spacers of 0.75, 1 or 1.5 mm. Cover the surface with ethanol 100 % and discard the ethanol after polymerization. The stacking gel, which is poured on top of the resolving gel, is composed of 5 % (v/v) acrylamide:bisacrylamide (30 % 37.5:1) supplemented with TCE diluted in 125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS. The polymerization is triggered upon addition of 0.1 % APS and 0.1 % TEMED and the comb is added right after pouring the gel.
6. Mini-PROTEAN® Tetra Vertical Electrophoresis Cell from BIO-RAD.
7. 0.2 µm PVDF membranes.
8. Trans-Blot Transfer Packs (BIO-RAD).
9. Trans-Blot Turbo™ transfer system from BIO-RAD.
10. Ethanol 100 %.
11. TBS-Tween20: 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.3 % (v/v) Tween20.
12. Skim milk powder.
13. Heidolph Duomax shaker.
14. Primary antibody (see **Table 2**).
15. Secondary antibody (see **Table 3**).
16. Clarity Western ECL substrate from BIO-RAD.

17. Chemidoc™ imaging system from BIO-RAD.
18. Red Ponceau.

3. Methods

3.1. Engineering the plasmid expressing the $GFP_{ß11ch}$-tagged gene of interest using the Gibson assembly procedure

1. PCR-amplify the gene of interest using the G1-for and G2-rev primer (see **Fig. 2** for details)

2. The isothermal assembly is performed as follows :11 µL 2× reaction master mix is added to 9 µL DNA solution that is classically obtained by mixing PCR fragments (1-5 fragments, approximately 1 µL each) and sterile ultapure water. The reaction mixture is immediately incubated at 50 °C for 30-60 min. Parental plasmid DNA present within PCR products can be eliminated *via* DpnI digestion (see **Note 3**). 5 µL of the whole mixture is used to transform chemically-competent *Escherichia coli* cells [10] and yields classically 10-800 colonies.

3.2. Transforming the BiG Mito-Split-GFP strain

1. Inoculate BiG Mito-Split-GFP Strain from a YPD rich medium plate in 3 mL liquid YPD medium and grow overnight (ON) at 30 °C under 200 rpm rotational shaking.
2. Pellet cells by centrifugation at 5,000 × *g* for 5 minutes (min) at room temperature (RT).
3. Heat the salmon sperm DNA (10 mg/mL) at 95 °C for 5 min to solubilize it.
4. Wash cells with 1 mL sterile ultrapure water and centrifuge at 5,000 × *g* for 5 min at RT.
5. Repeat step 4.
6. Resuspend the pellet in the appropriate volume of sterile ultrapure water (see **Note 4**).
7. In a 1.5 mL microtube add 10 µL of cooled salmon sperm DNA before adding 50 µL of cell suspension (see **Note 5**). Mix by vortexing for 5 seconds (s).

8. Add 1 µg of the p414-pGPD-GOI-GFP$_{ß11ch}$ (see **Fig. 2**) to the cells and then 72 µL sterile ultrapure water, 36 µL lithium acetate 1 M and 240 µL polyethylene glycol (PEG)-4000 50 % (w/v). Mix by vortexing for 5 s.
9. Incubate cells in a 42 °C water bath for 20 to 40 min.
10. Harvest cells by short spin centrifugation (approximately 10 s). Add 1 mL sterile ultrapure water (see **Note 6**), mix by inversion without resuspending the pellet and immediately remove the supernatant to leave 100 – 150 µL liquid in the tube.
11. Resuspend the pellet using the remaining supernatant.
12. Spread cells on a Petri dish containing solid SCD-Trp medium using sterile glass beads.
13. Incubate the plate at 30 °C for 3 – 4 days until colonies appear.
14. Streak colonies on a new SCD-Trp plate and incubate at 30 °C for 2 – 3 days.
15. Select the clones that can grow on SCD-Trp and store the SCD-Trp plate at 4 °C.

3.3. Culture of the transformed BiG Mito-Split-GFP strain

1. Grow 3 – 4 selected clones overnight (ON) in 3 mL liquid SCD-Trp medium at 30 °C under 200 rpm rotational shaking.
2. In the morning, dilute the cells to an $OD_{600\ nm}$ = 0.4 in 3 mL SC Galactose (SCGal)-Trp medium and incubate them at 30 °C under 200 rpm rotational shaking to an $OD_{600\ nm}$ = 0.8 – 1.2.
3. Separate equally the whole culture into two 1.5 ml sterile microtubes, one for the preparation of total protein extract and Western blot (to verify expression of the GFP$_{ß11ch}$-tagged protein; see **section 3.5.**) and the other one for mitochondria staining and microscopy imaging.

4. Centrifuge the cells for total protein extract at 5,000 × *g* for 5 min at RT and discard the supernatant.
5. Keep the cell pellet for total protein extract on ice and proceed with mitochondria staining and microscope imaging.

3.4. Visualizing mitochondrial importability of the GFP$_{ß11ch}$-tagged protein

1. Add MitoTracker™ Red CMXRos (Thermo Fisher) to a final concentration of 100 µM to the cells prepared at **section 3.3. step 3.** for mitochondria staining.
2. Incubate the cells at 30 °C for 15 min.
3. Centrifuge the cells at 5,000 × *g* for 5 min at RT.
4. Wash cells with 1 mL sterile deionized water.
5. Repeat **step 4.** twice.
6. Resuspend cells in 50 µL SCGal medium.
7. Drop 2.3 µL of stained cells between 76 × 26 mm microscope slide and 22 × 22 mm cover slip (see **Note 7**).
8. Perform epifluorescence imaging (see **Fig. 3**) using an AXIO Observer d1 (Carl Zeiss) epifluorescence microscope using a 100 × plan apochromatic objective (Carl Zeiss). The images are acquired using the GFP, TRITC and Nomarski filters and using the camera CoolSnap HQ2 photometrix (Roper Scientific). For image processing and montage, the software ImageJ is used.

3.5. Verifying expression of the GFP$_{ß11ch}$-tagged protein and/or of the mitochondrial GFP$_{ß1-10}$ fragment

1. Resuspend the pellet in 450 µL NaOH 0.185 M.
2. Incubate the suspension 10 min on ice.
3. Add 50 µL trichloroacetic acid 100 %.

4. Incubate the suspension 10 min on ice.
5. Pellet the precipitated proteins at 13,000 × *g* for 15 min at 4 °C.
6. Remove the supernatant (see **Note 8**) and resuspend the pellet in 75 µL Laemmli buffer 1 × (see **Note 9**).
7. Prepare polyacrylamide gels and load 7.5 µL of total protein extract in Laemmli buffer onto the polyacrylamide gel and perform the electrophoresis at 180 V for 10 min and then at 200 V using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell from BIO-RAD.
8. If TCE was added to the polyacrylamide gel, proteins having migrated can be detected using Stain-free procedure on Chemidoc™ imaging system from BIO-RAD (see **Note 2**).
9. Transfer proteins onto 0.2 µm PVDF membranes using Trans-Blot Transfer Packs and Trans-Blot Turbo™ transfer system from BIO-RAD (see **Note 10**).
10. Shortly incubate the membrane in ethanol 100 % and wash with 10 ml TBS-Tween20 solution.
11. Block the membrane with 10 ml TBS-Tween20 solution supplemented with 5 % (w/v) skim milk for 30 min at RT under shaking on Heidolph Duomax 1030 at 10 rpm. Discard the blocking solution.
12. Incubate the membrane overnight at 4 °C under shaking with 10 mL blocking solution containing the primary antibody (see **Table 2**) diluted 1:5,000 (see **Note 11**).
13. The next day, remove the primary antibody solution.
14. Wash the membrane with 10 ml TBS-Tween20 solution for 10 min at RT under shaking. Discard the washing solution.
15. Repeat step 14 two times

16. Add 10 mL of the secondary antibody (see **Table 2**) diluted 1:5,000 in blocking solution and incubate for 3 hours at RT under shaking.
17. Remove the secondary antibody solution.
18. Wash the membrane with 10 mL TBS-Tween20 solution for 10 min at RT under shaking.
19. Repeat step 18 twice.
20. Wash the membrane with 10 mL TBS 1 × solution.
21. Add the Clarity Western ECL substrate from BIO-RAD (750 µL of each reagent for one membrane).
22. Perform immunodetection using the Chemidoc™ imaging system from BIO-RAD and the Chemiluminescence program (manual acquisition and optimal time exposure).
23. Rinse the membrane with TBS-Tween20 solution shortly and add undiluted Red Ponceau staining to the membrane (see **Note 12**). Incubate 20 min at RT under shaking.
24. Remove the Red Ponceau staining solution and wash the membrane with 10 mL TBS-Tween20 solution until the red staining of the membrane disappear.
25. Start from step 11 using a second set of primary and secondary antibodies.

4. Notes

1. 4X Laemmli buffer: 0.5 M Tris-HCl pH 6.8, 8 % (w/v)SDS, 40 % (v/v) glycerol, 0.05 % (w/v) Bromophenol blue
2. 2,2,2-Trichloroethanol is added to the acrylamide:bisacrylamide 30 % (v/v) stock solution to visualize proteins that have migrated inside the gel by UV detection using the Stain-free procedure using a ChemiDoc Touch Imaging System. This procedure can serve as loading control to assess that all lanes were initially loaded with comparable amounts of proteins. Alternatively, Red Ponceau staining of the membrane can be performed after transfer to verify the migration and transfer.
3. In order to remove the parental plasmid DNA used as a matrix for PCR amplification, DpnI digestion can be performed. For this, the isothermal assembly reaction is supplemented with 2 µL of 10 × FastDigest buffer and 10 U (1 µL) DpnI (Thermo Fisher) and incubated at 37 °C for 1 h. Subsequently, DpnI is heat-inactivated (5 min at 95 °C). Removing the template DNA from the isoT reaction decreases the possibility to transform *E. coli* cells with an empty plasmid and thus the number of false positives.
4. The cell suspension should be milky without being too dense. Usually for a volume of 1 mL of an overnight culture, 100 µL deionized water are used to resuspend the pellet.
5. Make sure the salmon sperm DNA is not too hot when adding the cells to avoid heat shock, that would cause cell death and thus reduce transformation efficiency.
6. The pellet should not be resuspended when deionized water is added after heat shock. Addition of water will dilute the transformation mix.
7. To avoid bubbles, even in the middle of the coverslip, press on the corners. Pressing in the middle of the coverslip would cause cell burst.

8. Ensure to remove all the droplets when discarding the protein precipitation solution. Residual trichloroacetic acid droplets will change the blue color of Laemmli solution to a yellow/orange color. This indicates that pH of the sample is too acid which can cause troubles during SDS-PAGE electrophoresis.
9. If the solution has a yellow or orange color after adding the 1 × Laemmli solution to the cells, Tris-Base 1 M should be added until the color turns to deep blue (start using 1 µL Tris-Base and increase the volume if the color does not change). Laemmli buffer 1 × can also be supplemented with 1 M Tris-Base.
10. We used the Trans-Blot Turbo transfer system from BIO-RAD for our experiments, but any transfer system can be used.
11. The primary antibody can also be incubated at RT for 3 h under shaking. However, better results were obtained with an ON incubation at 4 °C.
12. Red Ponceau staining solution is used to denaturate the HRP coupled to the secondary antibody. By doing so, it is possible to incubate the membrane with another set of primary and secondary antibodies, without being disturbed by the HRP signal emitted by the first secondary HRP-coupled antibody. However, this method does not remove HRP-coupled secondary antibody from membrane. In order to reuse the membrane with a second set of antibodies, other stripping methods (heat and detergent, low pH or kits provided by different manufacturers) can be used.

5. References

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Figures & Legends

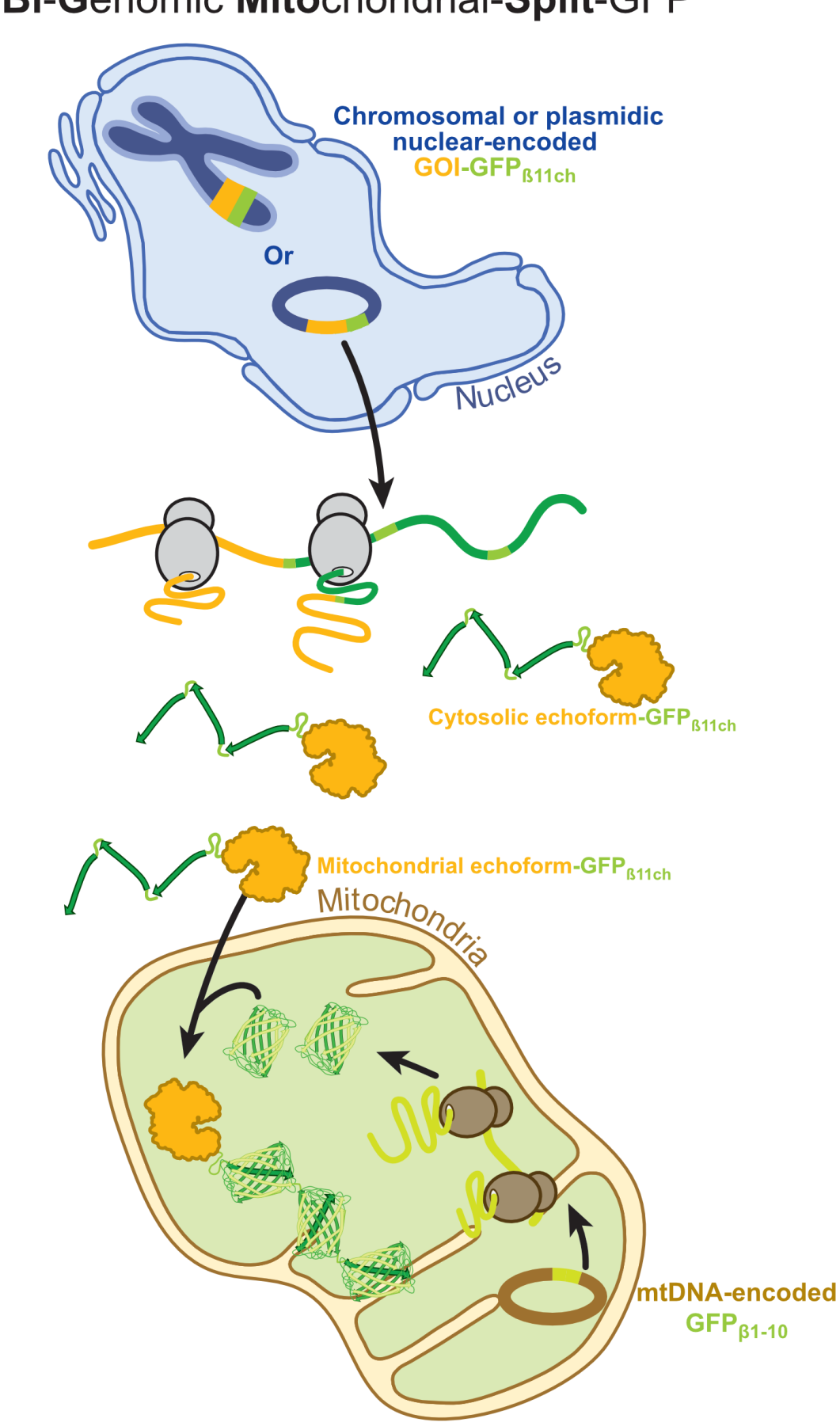
Bi-Genomic Mitochondrial-Split-GFP
Chromosomal or plasmidic nuclear-encoded GOI-GFPß11ch
Or
Nucleus
Cytosolic echoform-GFPß11ch
Mitochondrial echoform-GFPß11ch
Mitochondria
mtDNA-encoded GFPβ1-10

Fig. 1.

Fig 1. Principle of the BiG Mito-Split-GFP system. The gene of interest (GOI) encoding a dual-localized protein has been tagged at its 3'-end with the sequence of three interspaced ß11 fragments of the Split-GFP (ß11chaplet; $GFP_{ß11ch}$). The mRNA of this $GOI_{ß11ch}$ gene will be translated by cytosolic ribosomes into the corresponding protein fused at its C-terminus with the ß11ch tag. The pool corresponding to the cytosolic ß11ch-tagged echoform stays in the cytoplasm and is thus not generating any fluorescent signal, while the mitochondrial ß11ch-tagged echoform will translocate inside the mitochondrial matrix. Upon mitochondrial import the ß11ch appended to the mitochondrial echoform will bind to $GFP_{ß1-10}$ fragment of the Split-GFP translated by mitoribosomes from transcripts transcribed from a mtDNA-integrated ß1-10 gene. Upon interaction both mitochondria-restricted Split-GFP fragments will reconstitute a functional GFP yielding fluorescent restricted to mitochondria that can be visualized by epifluorescence microcopy.

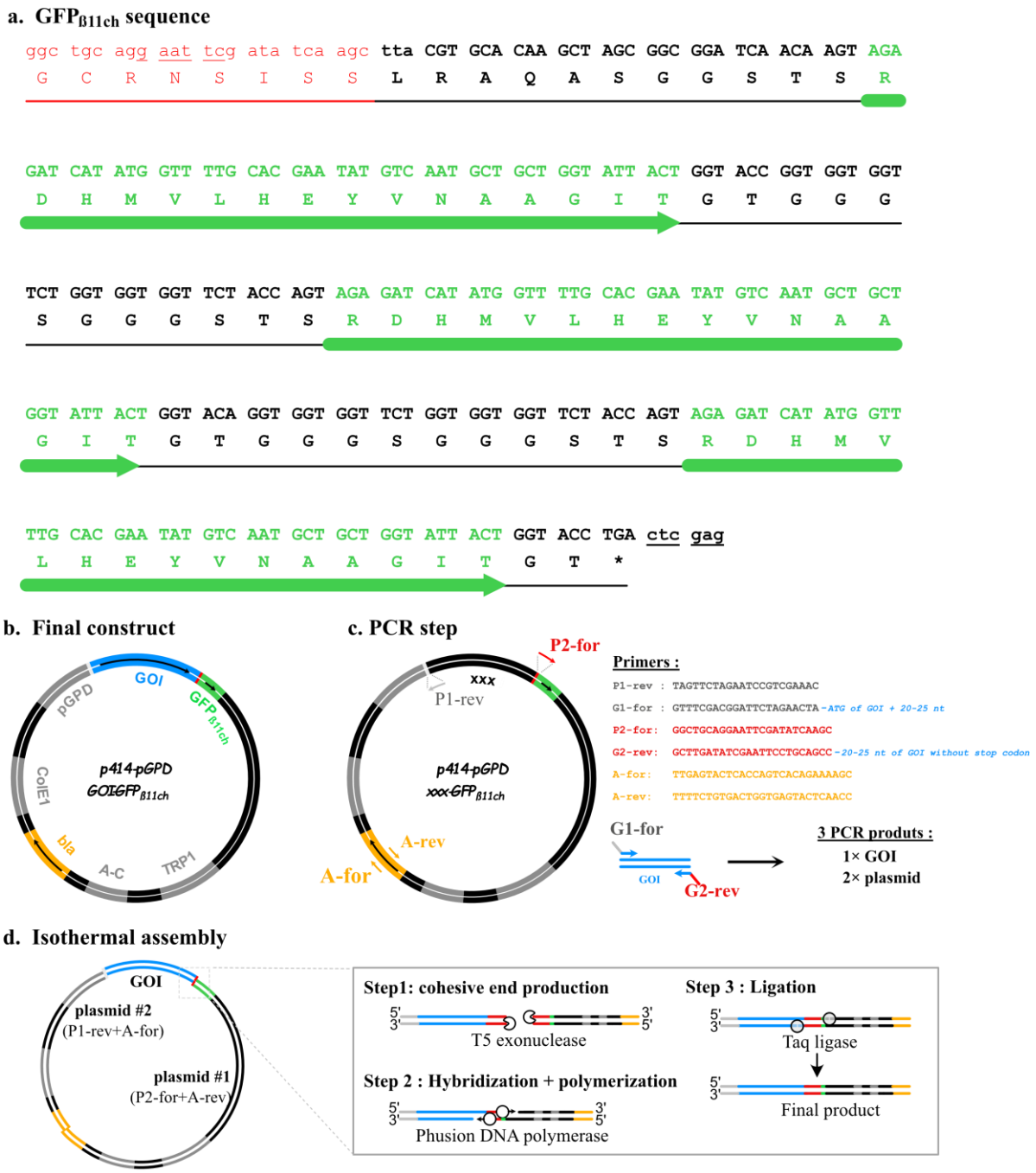

Fig. 2.

Fig 2. Adding the GFPß11ch tag (**a**) at the 5'-end of gene of interest using the isothermal assembly strategy. (**a**) Sequence (Nucleotides and amino acids) of the GFPß11ch tag. The ß-strands (arrows) of the GFPß11ch tag are in green and the spacers between the ß11 fragments are in black. Nucleotides in red correspond to the downstream isoT tag (used for P2-for and G2-rev). Note that underlined nucleotides show EcoRI (red) and XhoI (black) restriction sites

at both ends of the $GFP_{ß11ch}$. (**b**) *In silico* assembly of the desired construct (p414-pGPD-GOI-$GFP_{ß11ch}$) showing showing the selection marker (bla (ampicillin resistance), yellow), the gene of interest (GOI, blue), the $GFP_{ß11ch}$ tag (green) with a small linker region (red), the GPD promoter (pGPD), the yeast auxotrophy marker TRP1, the *E. coli* origin of replication (ColE1) and the yeast centromeric origin of replication (A-C : ARS/CEN). (b) To obtain the final construct, PCR amplification is used for the GOI and the two plasmidic fragments. The template used to amplify the destination vector is a similar plasmid containing an irrelevant GOI (named xxx here). The position and the sequence of the primers is indicated. Please note that for primer G2-rev the reverse-complement sequence of your GOI has to be considered (without stop codon) since the primer has the same sequence than the minus strand of the GOI. (c) Gibson assembly of the 3 PCR products (the GOI and the two plasmid halves). This assembly is performed at 50°C and has three steps : i) T5 exonuclease (5'→ 3') creates short 3' overhangs since it gets rapidly inactivated at 50°C, ii) the single stranded overhangs can hybridize and become substrates for Phusion DNA polymerase and iii) the fragments are ligated together by Taq DNA ligase to yield the final product.

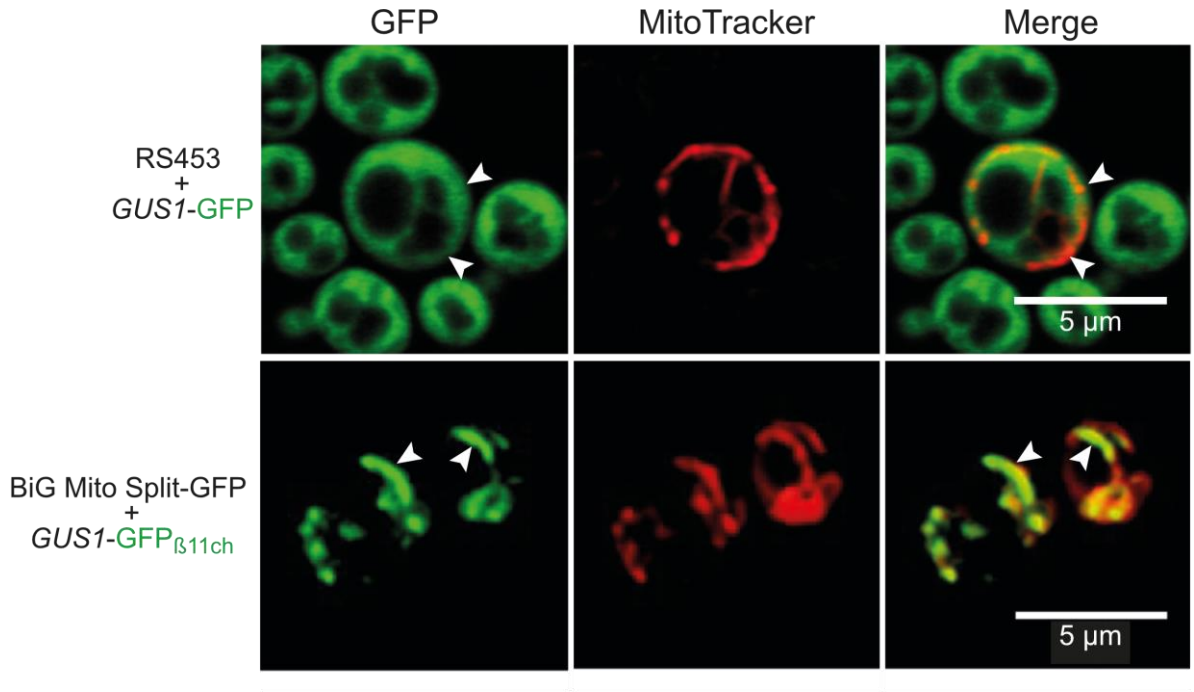

Fig. 3.

Fig 3. **Fluorescence emission patterns of a dual-localized protein ß$_{11ch}$ tagged and expressed in the BiG Mito-Split-GFP to that of the same protein tagged with regular GFP.** Micrographs of the RS453 strain expressing *GUS1* tagged with regular GFP (top) and of the BiG Mito-Split-GFP strain expressing *GUS1* tagged with ß11$_{ch}$ (bottom). GUS1 encodes glutamyl-tRNA synthetase which has been shown to be dual-localized both in the cytosol and the mitochondria [11]. Cells were treated as described in **section 3.4. steps 1-7** and images were taken as described in **section 3.4., step 8** and processed with the ImageJ software. Mitochondrial echoforms of glutamyl-tRNA synthetase are indicated with white arrowheads.

Tables

Table 1: BiG Mito-Split-GFP strain genotypes

Strain	Nuclear genotype	Mitochondria DNA

BiG Mito-Split-GFP	*MATa his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::HIS3*	ρ^+ *atp6::GFP*$_{ß1\text{-}10}$ *5'UTR*$_{COX2}$ *ATP6 3'UTR*$_{COX2}$

Table 2: Primary antibodies used in this study

Specificity	Name	Type	Vendor	Dilution
$GFP_{ß1\text{-}10}$	Anti-GFP N-terminal	Rabbit polyclonal	Sigma (#G1544)	1:5,000
$GFP_{ß11ch}$	Anti-GFP	Mouse monoclonal $IgG_1\kappa$ clones 7.1 and 13.1	Roche (#11814460001)	1:5,000

Table 3: Secondary antibodies used in this study

Targeted primary antibody	Type	Dilution
Anti-GFP N-terminal	Goat-Anti-rabbit-HRP	1:5,000
Anti-GFP	Goat-Anti-mouse-HRP	1:5,000