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Photoactivatable green fluorescent protein-based visualization and quantification of mitochondrial fusion and mitochondrial network complexity in living cells

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Abstract

Technological improvements in microscopy and the development of mitochondria-specific imaging molecular tools have illuminated the dynamic rearrangements of these essential organelles. These rearrangements are mainly the result of two opposing processes: mitochondrial fusion and mitochondrial fission. Consistent with this, in addition to mitochondrial motility, these two processes are major factors determining the overall degree of continuity of the mitochondrial network, as well as the average size of mitochondria within the cell.

In this chapter, we detail the use of advanced confocal microscopy and mitochondrial matrix-targeted photoactivatable green fluorescent protein (mito-PAGFP) for the investigation of mitochondrial dynamics. We focus on direct visualization and quantification of mitochondrial fusion and mitochondrial network complexity in living mammalian cells. These assays were instrumental in important recent discoveries within the field of mitochondrial biology, including the role of mitochondrial fusion in the activation of mitochondrial steps in apoptosis, participation of Bcl-2 family proteins in mitochondrial morphogenesis and stress induced mitochondrial hyperfusion. We present some basic directions that should be helpful in designing mito-PAGFP-based experiments. Furthermore, since analyses of mitochondrial fusion using mito-PAGFP-based assay rely on time-lapse imaging, critical parameters of time-lapse microscopy and cell preparation are also discussed.

Keywords

mitochondria; fusion; photoactivation; green fluorescent protein; time-lapse imaging; mitochondrial network

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A) Introduction

Mitochondria are intracellular organelles key to the physiology of eukaryotes. They provide energy to cells through the conversion of carbon sources into ATP, function in iron and calcium homeostasis and play a central regulatory role in programmed cell death. The dynamic nature of mitochondria in eukaryotic cells has been apparent since the advent of light microscopy in the early 20th century (Lewis and Lewis, 1914). Recent advancements in microscopy and the development of mitochondria-specific fluorochromes have illuminated the dramatic rearrangements of shape that mitochondrial membranes undergo within a single cell in short time intervals (Bereiter-Hahn and Voth, 1994).

Depending on the cell type, physiological process and metabolic requirements of the cell, mitochondria exist in different shapes and quantities. The two most common morphologies of mitochondria are 1) long, filamentous networks and 2) short, grain-like mitochondria, and these forms occur in a dynamic equilibrium within a single cell. This equilibrium exists as mitochondria are continuously undergoing the opposing processes of fusion and fission. In addition to the creation of new mitochondrial material, the relative contribution of each process determines the overall degree of continuity of the network, as well as the average size of mitochondria within the cell (for reviews see (Benard and Karbowski, 2009; Chan, 2012; Meeusen and Nunnari, 2005; Nunnari and Suomalainen, 2012). Fission and fusion of membranes are common within cells, but these processes are inherently more complex in mitochondria due to their double membrane structure.

Mitochondria have two structurally distinct membranes: the outer membrane (OMM) and the inner membrane (IMM). Together they define two compartments: the intermembrane space between the two membranes and the mitochondrial matrix enclosed within the inner mitochondrial membrane. While the OMM is relatively smooth, the IMM contains folds called cristae that serve to increase the surface area for proteins involved in the electron transport chain. Although work in yeast has demonstrated that the fusion of the outer and inner membranes is temporally linked *in vivo*, they can be separated (Meeusen and Nunnari, 2005). This separation has led to the identification of different requirements for the fusion of each membrane. The regulation of mitochondrial fusion events is cell type dependent in mammalian cells, as structure and dynamics of these organelles differ between tissues.

Most of the proteins involved in these pathways were identified in genetic screens in the budding yeast, S*acchromyces cervisiae*, based on defects in mitochondrial shape that occur in yeast mutants. Interestingly, three of the key proteins involved in these dynamic and opposing processes are conserved large GTPases. Fzo (Fzo1p in yeast, Mfn1 and Mfn2 in mammals), first identified in *Drosophila melanogaster* (Hales and Fuller, 1997), is a large GTPase essential for mitochondrial fusion (Chen et al., 2003; Hermann et al., 1998; Santel and Fuller, 2001). Fzo1p is localized to the outer mitochondrial membrane by two transmembrane domains. The second GTPase involved in mitochondrial fusion is Mgm1p (Opa1 in humans), and it has also been shown to participate in cristae remodeling and maintenance of mitochondrial DNA (Guan et al., 1993; Shepard and Yaffe, 1999; Wong et al., 2000). Mgm1p is associated with the inner mitochondrial membrane and is postulated to regulate fusion of inner membranes. It is also possible that this protein plays multiple roles,

with at least two proteolytically generated isoforms in yeast and multiple splice variants in mammals. Post-translational modification of the basic fission and fusion machinery expands the pool of proteins involved in regulating mitochondrial morphology as well. For example, Opa1 has long been appreciated to undergo proteolytic cleavage into various isoforms, with recent work linking this processing to mitochondrial fission and fusion (Anand et al., 2014; Meeusen and Nunnari, 2005).

In addition to the fusion proteins discussed above, Dnm1p (Drp1/Dlp1 in mammals) is a large GTPase with a key role in mitochondrial fission [(Bleazard et al., 1999; Smirnova et al., 2001); for reviews see (Benard and Karbowski, 2009; Chan, 2012; Nunnari and Suomalainen, 2012)]. While Dnm1p is assembled into punctate structures on the outer mitochondrial membrane in yeast (as is Drp1 in mammals), it can also be found in the cytoplasm. Time-lapse imaging of this fission protein has demonstrated that these punctate foci are also scission sites on mitochondrial tubules [reviewed in (Benard and Karbowski, 2009; Chan, 2012; Nunnari and Suomalainen, 2012)]. Genetic screens in yeast have identified Mdv1p and Fis1p as other proteins that interact with Dnm1p during fission of mitochondria (Mozdy et al., 2000; Tieu and Nunnari, 2000). Homologs of Mdv1p have not been identified in higher eukaryotes, but Fis1p homologs exist in a wide range of eukaryotic organisms, including worms and mammals [reviewed in (Benard and Karbowski, 2009; Chan, 2012; Nunnari and Suomalainen, 2012)]. In addition to Drp1 and Fis1 in higher order eukaryotes, several other postulated Drp1 receptors exist on the outer mitochondrial membrane, including Mff, MiD49 and MiD51/MIEF1 [reviewed in (Chan, 2012)].

The refinement of confocal microscopy techniques and the discovery and development of fluorescent protein markers from marine organisms, such as green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, have greatly transformed cell biology. Importantly, these advances were instrumental in understanding of the mechanisms of mitochondrial fission and fusion. Consistent with this, most of the discoveries described above were greatly facilitated by the combined use of confocal imaging and GFP-based probes.

Recent advances in understanding mitochondrial fission and fusion have been made possible by the expansion of GFP and related technologies. Many GFP mutants have been engineered to improve brightness, expression and photostability, while minimizing dimerization. The option to use different colors for multi-label experiments is also available. In addition, a photoactivatable variant of GFP (PAGFP) was developed in 2002 (Patterson and Lippincott-Schwartz, 2002) with the aim of optimizing the photoconversion properties of this fluorescent molecule. Through a mutation of threonine 203 to histidine, the basal absorbance of this protein was decreased, allowing for the fluorescence to be “switched on”. Photoactivation by ~400-nm light leads to a 100-fold increase in fluorescence intensity, allowing one to follow the movements of individual pools of labeled proteins *in vivo*. Fluorescent protein-tagged proteins and time-lapse confocal microscopy are invaluable for the study of certain dynamic cellular events, and have been particularly useful in dissecting mitochondrial dynamics. The kinetic properties of a protein, such as whether it is freely diffusible, bound to an immobile complex or membrane, or undergoing stages of binding and dissociation with other components within a cell, underlie its function *in vivo*. Thus, techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence (or

Förster) resonance energy transfer (FRET), or photoactivation have been developed for use in combination with confocal time-lapse microscopy to better understand various properties of cellular proteins and organelles [for reviews see (Day and Davidson, 2012; Hitchcock et al., 2006; Ward and Lippincott-Schwartz, 2006)].

In this chapter, we will discuss the application of confocal microscopy and mitochondrial matrix-targeted PAGFP (mito-PAGFP) for the investigation of mitochondrial dynamics with a focus on direct visualization and quantification of mitochondrial fusion and mitochondrial network complexity in living cells.

B) Visualization and quantification of mitochondrial fusion in living cells

Mitochondria in cells fuse and fragment continuously. Identification of proteins that participate in the mitochondrial fusion process requires analysis of the physiological effects induced by the inhibition or knock out of these proteins. An RNAi screen of mitochondrial genes in *C. elegans* indicated that nearly 80% of the knockdowns displayed altered mitochondrial morphology towards fragmentation and/or aggregation (Ichishita et al., 2008). It is unlikely that all of these regulate mitochondrial fusion, as the final morphology may reflect an increase in fission, a decrease in fusion or an inhibition of biogenesis. Thus, it is important to assess rates of fusion when such morphologies are observed.

Isolated mitochondrial preparations can be used to assess mitochondrial fusion *in vitro*. These assays provide many benefits including robust control over conditions and limited interference from unknown cellular factors. These assays were initially characterized in yeast and then used to analyze yeast homologues of the fusion machinery in higher organisms (Meeusen et al., 2004; Meeusen and Nunnari, 2007). These approaches rely on successful isolation of healthy mitochondria from living cells however and the benefit of robust control over experimental conditions can be a drawback when an understanding of fusion in the light of cellular context is desired.

Until recently, mitochondrial fusion in intact yeast and mammalian cells could only be assayed using cell fusion-based assays, like the polyethylene glycol (PEG) assay. In this approach, cells stably expressing mitochondria-targeted GFP are co-plated with cells stably transfected with mitochondria-targeted red fluorescent protein (RFP). Cell fusion is then induced by PEG treatment, followed by analysis for GFP-RFP mixing in fused polycaryons. Formation of “yellow” mitochondria containing both fluorophores indicates that matrix compartments of mitochondria expressing green and red markers had mixed their contents, indicating mitochondrial membrane fusion (Chen et al., 2003; Ishihara et al., 2003; Legros et al., 2002). Although this method can give some approximation of mitochondrial fusion dynamics, it cannot be used to analyze this process in a majority of cell types, including primary cells, nor can it be utilized for mitochondrial fusion studies in real time (e.g. following mitochondrial dynamics in cells primed for apoptotic cell death). Described here is an alternative assay for mitochondrial fusion that enables real-time analysis of this process in any cell type that can be transfected, including cell types that cannot be induced to fuse with PEG (e.g. cultured neurons and other primary cell types) (Karbowski et al., 2004). In this approach cells are transfected with a construct containing mitochondrial matrix targeted

photoactivatable green fluorescent protein (mito-PAGFP). In a mito-PAGFP-based mitochondrial fusion assay, the regions of interest (ROIs) within cells expressing mito-PAGFP are excited briefly with a ~400-nm laser to activate selected pools of mitochondrial matrix localized PAGFP, followed by time-lapse 488-nm imaging of activated cells. The redistribution and dilution of mito-PAGFP fluorescence from the matrix of a "photoactivated" mitochondrion into non-activated ones that occurs as a result of mitochondrial membrane fusion is used to monitor and quantify this process. The methodology discussed here has been validated using various cell types, including fusion deficient Opa1-, Mfn1- and Mfn2-depleted cells (Karbowski et al., 2004; Karbowski et al., 2006; Lee et al., 2004) (Fig. 1) and should result in efficient visualization of mitochondrial fusion in real time, as well as quantification of fusion rates in variety of cells under different experimental conditions. The general steps of the assay that can be used as a reference for designing cell-specific mito-PAGFP-based mitochondrial fusion assay are described below.

Materials

Chamber slides (for example Lab-Tek brand chambered coverglass with cover, #1 German borosilicate; Nalge Nunc International), or any other cell culture vessels with glass bottoms appropriate for confocal microscopy imaging.

Confocal microscope—An objective with the highest possible numerical aperture should be used to facilitate the greatest collection of emission light (brightness). We have found that 100x Plan-Apochromat lenses with a numerical aperture of 1.45 give optimal brightness and resolution. However, 63x Plan-Apo objective lenses with a numerical aperture 1.4 are also suitable.

Procedure

1. Transfect cells with mito-PAGFP construct. As mito-PAGFP is barely detectable before photoactivation (see Fig. 1 "preactivation" images) it might be difficult to identify cells transfected with this construct. If this is the case cells could be cotransfected with an additional mitochondrial matrix targeted, preferably red fluorescent, construct (e.g. mito-DsRED2; or if cells do not tolerate high ectopic expression of protein targeted into the mitochondrial matrix it can be replaced with DsRED2 fused to a nuclear targeting sequence, nuc-DsRED2) at the ratio 3:1 of mito-PAGFP: DsRED2 construct. The higher amount of photoactivatable GFP used for co-transfection will assure that this protein is present in cells expressing DsRED2. Also, the time between transfection and analysis should be carefully optimized. We found that in most cells sufficient expression of mito-PAGFP is apparent as early as ~12hr after transfection. However, some primary cells (e.g. hippocampal neurons) as well as some lineages of immortalized mouse embryonic fibroblasts may require ~24-36hr for optimal mito-PAGFP expression.
2. If mito-PAGFP cotransfection with mito-DsRED2 is used the cells expressing moderate levels of mito-DsRED2 should be imaged with a low power level for 543-nm and 488-nm lasers. The multiplier gain should be adjusted to obtain signal

intensity of mito-DsRED2 slightly below the saturation level; the green channel should be set to achieve a barely visible signal of non-activated mito-PAGFP.

3. Select a region of interest to be photoactivated. Using the image obtained in the previous step, create a circular ROI within the mitochondrial network (a red channel image of mito-DsRED2 can be used for the reference) with a diameter corresponding to several mitochondrial units (single mitochondria). The region to be photoactivated should be relatively small compared to the size of mitochondrial network. We found that ROIs with diameters between 2μm and 5μm are optimal to assure that only single or a few bundled mitochondria are photoactivated. For the fusion quantification experiments, especially when using relatively large, flat cells (e.g. HeLa, U2OS or some lineages of MEFs), ROIs used for photoactivation should be preferentially located in the perinuclear area, where the density of mitochondria is high, enabling randomization of each measurement. This will also eliminate potential variability that can occur due to higher spatial separation of mitochondria detectable in the periphery of several cell types. When using polarized cells (e.g. cultured neurons) it is possible to measure the fusion rates in different cell sub-compartments (e.g. cell body versus processes).

4. Photoactivate selected ROIs with a short pulse of ~400-nm laser and obtain a series of images. Collect a pre-activation image as in step 2, position the activation ROI within the mitochondrial network (step 3) and irradiate this area with the brief pulse of ~400-nm light. PAGFP absorbs predominately in the ultraviolet spectral region with a maximum centered around 395 to 400 nanometers. Illumination of the protein with light in the 400-nm region transforms the neutral phenolic chromophore into an ionic phenolate species that absorbs with a maximum at 483 nanometers. The photoconversion reaction is mediated by decarboxylation of the glutamate residue at position 222 in the fluorescent protein backbone. Thus, photoactivation of mito-PAGFP can be achieved with essentially any ~400-nm light source. The best results in our laboratory were obtained with 405-nm laser irradiation; however, sufficient photoactivation was also achieved with a 413-nm laser, and two-photon laser set at 800-nm.

Before collecting data it is critical to carefully determine the minimal time of each laser scan, number of iterations, and the power of laser that may be required for a several fold increase of the fluorescence within and in close proximity of photoactivated ROIs (see Fig 1, yellow circles in “detail” images). This is one of the most critical steps in this method. Mitochondria are very sensitive to photo damage, and prolonged illumination with ~400-nm light can induce rapid fragmentation of the mitochondrial network. Furthermore, mitochondrial network complexity should also be considered when deciding on the number of iterations necessary for adequate photoactivation. Photoactivation of mito-PAGFP within low complexity, small or fragmented mitochondria will generally require less excitation light. Quantitative analysis of mitochondrial matrix-localized GFP (mito-GFP) spot photobleaching data determined diffusion coefficients of 2-3 × 10^{-7} cm^2/s, only three to fourfold less than that for GFP diffusion in water (Partikian et al., 1998). While the diffusion rates of mito-PAGFP have not been measured, it is likely that they are similar to those of mito-GFP. Due to the fast diffusion of GFP within the mitochondrial matrix, photoactivated

PAGFP rapidly diffuses from the activated ROIs. This results in fluorescence dilution, especially in larger or more interconnected mitochondria. Thus, this step should be very carefully optimized. Following this optimization, the following series of images should be acquired: (1) a preactivation image and (2) a number of images taken at different time points after photoactivation, with at least one image obtained immediately after activation. We found that in most cell types distinct redistribution of photoactivated mito-PAGFP from "activated" to "non-activated" mitochondria can usually be observed within 10-45 minutes after photoactivation (Fig. 1B). So for the measurements of mitochondrial fusion it is sufficient to collect a series of images consisting of an immediate post-activation image (e.g. at 30 sec) and images taken at 15 min, 30 min and 60 min post-activation. These intervals are applicable to analysis of samples predicted to have major differences in mitochondrial fusion rates. However, shorter intervals should be used in experiments in which less pronounced changes/differences in mitochondrial fusion rates are expected (e.g. when comparing different cell types).

Most confocal microscopes are equipped with optional automatic computer-regulated stages. With these it is possible to activate and record several cells during each experiment. For example, the stage control software supplied with a Zeiss LSM710 imaging system allows the positions of several tens of cells to be marked and programmed to return to specific cells at the appropriate time post-activation, maximizing the data obtained in one experiment. In order to avoid false-positive decreases in the mito-PAGFP fluorescence that can occur when activated mitochondria move out of the imaging plane, when possible stacks of z-sections covering the entire thickness of the cell should be acquired. However, to minimize phototoxicity, acquiring three optical sections (top, middle, and bottom of the cell) should be sufficient. This will lead to visualization of all mitochondria in an analyzed cell. When imaging single events of mitochondrial fusion, a time-lapse series with a maximum interval of 1 min can be collected.

5 Measurements of the redistribution rate of mito-PAGFP from activated into non-activated mitochondria can be used as a relative rate of mitochondrial fusion. Images transferred into image analysis software such as ImageJ or Metamorph can be analyzed using the region measurement tool to collect pixel intensities within activated ROIs as well as in non-activated areas of the cell from all images in the series. The obtained values can then be plotted as a function of time. Analyses of several cells in each experimental group should efficiently eliminate experimental condition-induced (e.g. mito-PAGFP expression level variance) variability. Furthermore, to reduce acquisition of nonspecific fluorescence, only images showing at least 10-fold post activation (at 30sec) increases in mito-PAGFP fluorescence signals within the activated ROI are suitable for analysis. Collected data can then be normalized, setting the preactivation value as 1. Calculation of averages and standard deviations should also be performed.

C) Estimation of relative size of mitochondrial units and mitochondrial network complexity

In recent years, changes in mitochondrial size and network organization have been linked to a variety of diseases and are also observed in a number of experimental models [for reviews see (Benard and Karbowski, 2009; Berman et al., 2009; Chan, 2012; Meeusen and Nunnari, 2005; Nunnari and Suomalainen, 2012)]. The most common way of analyzing mitochondrial size is a direct measurement of these organelles using images obtained from cells stained with mitochondria-specific fluorescent probes (either fluorescent dyes, including Mitotracker probes and TMRE, or mitochondria specific antibodies). While this method enables estimation of mitochondrial length, it also has several potential disadvantages. For example, immunofluorescence labeling of mitochondria results in visualization of all mitochondria within cells. Thus, in many cases it is extremely difficult to verify whether a measured mitochondrion is indeed a single organelle or whether it consists of several mitochondrial units localized in close proximity. Local photoactivation of mito-PAGFP is an alternative method to direct measurements of mitochondria (Karbowski et al., 2007). In this assay 2-4-μm wide rectangular ROIs are briefly irradiated with a ~400-nm laser followed by imaging with a 488-nm laser immediately after 400-nm photoactivation. This results in a photoactivation of mito-PAGFP within the ROIs. 488-nm confocal imaging performed immediately after ROI photoactivation reveals the area of mitochondrial network units located within the ROIs and those with matrix compartments contiguous with the ROIs. To quantify the relative area covered by ROI-activated mitochondria the whole imaging field can be subsequently photoactivated. This results in depiction of all the mitochondria within the same cells (Karbowski et al., 2007). The quantification of the ratio of area covered by ROI-activated mitochondria (r) to that covered by the whole cell mitochondria (w) should reveal relative mitochondrial unit sizes. The choice of parameters for image acquisition should follow the basic rules described for the mitochondrial fusion assay. However, while circular ROIs were used for analyses of mitochondrial fusion, we typically use rectangular ROIs covering the whole length of the cells for the mitochondrial size assay. When comparing mitochondrial network complexities in experimentally modified cells of the same type, photoactivation areas should be optimized using control cells within the group. For experiments comparing mitochondrial networks from different cell types, settings for a reference cell type can be used. We recommend the use of HeLa cells as a reference cell line; their mitochondrial network interconnectivity and length of single mitochondrial units is intermediate (Fig 1B)(Karbowski et al., 2004; Karbowski et al., 2007), making it an equally good reference point for cells with less or more elaborated mitochondrial networks.

D) Time-lapse imaging of mitochondria in living cells: general considerations

Since analyses of mitochondrial fusion rely on time-lapse microscopy imaging of mito-PAGFP, critical parameters of time-lapse microscopy and cell preparation are briefly discussed in this section. Cells should be transfected using well-optimized, cell-specific transfection protocols. The important parameters that should be considered include DNA concentration and the amount of transfection reagent necessary to achieve the expected

protein expression levels within a single cell, as well as the relative number of transfected cells. While these parameters vary between reagents and transfection techniques, they should be specified in the manufacturer's brochure for the transfection product. In general, it is critically important to evaluate the amount of DNA used for transfection and the time needed for expression of analyzed proteins. For example, a too high level of protein expression, either due to unnecessarily high levels of DNA or delayed analyses, could lead to artifactual changes in mitochondrial structure resulting in part from high protein-overexpression-induced abnormal mitochondrial fusion and fission rates. A concentration of DNA that results in acceptable transfection efficiency without affecting mitochondrial structure in a majority of cells should be used for transfection. The effect of transfection and protein expression on mitochondrial structure can be easily verified by comparing the transfected cells with untransfected cells immunolabeled to detect mitochondria. In our lab we use either anti-Tom20 (clone FL-145; Santa Cruz Biotechnology) or anti-cytochrome c antibody (clone 556432; BD Pharmingen) to detect the outer mitochondrial membrane or intermembrane space of the mitochondria, respectively. For transfection we most commonly use FuGENE6 (Roche) a multi-component lipid-based transfection reagent that forms a complex with DNA, allowing transport of the foreign DNA into the cell. We found that most of the cell types (including HeLa, U2OS, HCT116, and some lineages of mouse embryonic fibroblasts) transfected with this reagent can be used for analysis at 12hr after transfection. There are several specific considerations for transfection conditions to study mitochondrial dynamics. For instance, one of the most commonly used markers of mitochondria to date is mito-YFP. Like mito-PAGFP, this construct has a mitochondrial targeting sequence (MTS) attached to the sequence encoding yellow fluorescent protein (YFP). Once inside the mitochondrial matrix, the MTS is cleaved and the YFP acts as a soluble marker of the matrix. This protein expresses very well, and thus should be transfected at very low quantities. We found that in most cases FuGENE6 transfection with 0.1μg mito-YFP DNA per 1mL media in dish/chamber slide resulted in sufficient expression of mito-YFP, with negligible effects on mitochondrial structure when analyzed at ~12hr after transfection. Another mitochondrial marker that can be transiently transfected into cells is red fluorescent mitodsRed2, which is the same as mito-YFP only with dsRed2 as the soluble marker of the matrix. mito-dsRed2 should also be transfected in low amounts (i.e. 0.1μg of mito-dsRed2 DNA for every 1 mL media in dish or chamber slide of cells). We also recommend using similar amounts of DNA for mito-PAGFP transfection. In some cell types, mito-dsRed2 has been shown to disrupt mitochondrial morphology, so it is important to be familiar with the morphology of mitochondria in the cell type of interest in order to notice any inappropriate alterations. Furthermore, several recently developed GFP-variants and other fluorescent proteins can probably be used for mitochondrial time-lapse experiments, once suitable conditions are determined.

Specific analyses of the dynamic rearrangements of the outer mitochondrial membrane are also possible through the utilization of outer mitochondrial membrane-targeted fusion proteins. In our laboratory YFP fused with the OMM-targeting C-terminal 21 amino acid membrane anchor of Bcl-xL (the OMM-associated Bcl2 family protein) or OMP25 (the OMM associated protein with not well characterized function) proteins were successfully used. They are efficiently targeted to the OMM without detectable effects on mitochondrial

structure, even at relatively high expression levels. However, in general, proteins targeted to the OMM are more likely to change mitochondrial structure and fusion/fission rates, compared to those targeted to the mitochondrial matrix. Consistent with this, several other proteins tested by us (e.g. YFP fusions of Fis1, Mfn1, Mfn2 and TOM20) induced reorganization of mitochondrial structure even at relatively low expression levels. For example, overexpression of one of the fusion proteins, Mfn2, will often cause clumping of the mitochondria, making individual mitochondrial units difficult to identify visually. However, other labs have used these constructs for time-lapse imaging, suggesting that further optimization of transfection conditions or perhaps cell types used for the experiments are important factors. In general, cells expressing moderate levels of the labeled protein of interest should be selected for imaging. It is also recommended comparing cells expressing similar levels of fusion proteins, including mito-PAGFP. It is possible to combine live imaging of two or more fusion proteins (e.g. proteins stably associated with mitochondria together with protein transiently interacting or redistributing to mitochondrial membranes upon specific treatments). Spectral properties of fluorescent proteins used should be carefully considered, and combinations with the highest wavelength separations should be applied to avoid bleed-through from imaging one protein over other.

Finally, mitochondria are extremely sensitive to phototoxicity. Importantly, mitochondrial fragmentation, likely resulting from reduced mitochondrial fusion rates, is among the first factors induced by excessive cell illumination, even under relatively low excitation light intensity. Thus, finding the best balance between image resolution and cytotoxicity is of the most importance for successful time-lapse experiment. Consistent with this, the optimal conditions enabling prolonged imaging of living cells should be carefully optimized. Standard precautions applicable to any live cell imaging protocol, including minimization of exposure times, excitation light intensity, and overall imaging time, should be considered. There are several potential strategies to minimize the amount/intensity of excitation light necessary to collect clear data. For example, to obtain the best images without induction of severe phototoxicity, one can use reduced image size. In our lab we usually use 512×512 pixels. Furthermore, reduction of the frame averaging number and increase in pinhole size could be also used. While these approaches will reduce image resolution, they should still enable clear visualization of mitochondrial dynamics in living cells. Finally, higher NA objective lenses will collect more of the fluorescence, and they should be used in live cell imaging applications.

E) Concluding remarks: other potential applications of mito-PAGFP

Since the original publication (Karbowski et al., 2004), the mito-PAGFP-based mitochondrial fusion imaging method has been applied to various cell types, including skeletal muscle (Liu et al., 2014), control and LPS+IFNγ-treated astrocytes (Motori et al., 2013), cortical (Berman et al., 2009) and hippocampal neurons (Karbowski et al., 2004), *C. elegans* (Rolland et al., 2013) and many others. This assay was instrumental in several important discoveries within the field of mitochondrial biology, including the role of mitochondrial fusion in activation of mitochondrial steps in apoptosis (Karbowski et al., 2004; Lee et al., 2004), participation of Bcl-2 family proteins in mitochondrial morphogenesis (Berman et al., 2009; Karbowski et al., 2006; Perciavalle et al., 2012), stress

induced mitochondrial hyperfusion (Tondera et al., 2009), and the recently discovered, novel role of the mitochondrial fusion protein Opa1 in the control of mitochondrial division (Anand et al., 2014). Thus, while this work gives some basic directions that should be helpful in designing mito-PAGFP-based experiments, particular cell type specific conditions of the assay can be further determined using published reports. While most of the mitochondrial fusion research has been performed using established cell lines, further understanding of mitochondrial dynamics, including mitochondrial fusion, in primary cells derived from different tissues is important. Considering the well-established applicability of this mito-PAGFP-based fusion assay for almost any cell type, application of this method to currently uncharted cellular models will likely lead to further understanding of the scope and physiological importance of mitochondrial fusion. Furthermore, we believe that this straightforward and highly revealing assay can be applied to investigations of other areas besides mitochondrial fusion and mitochondrial network complexity. For example, since photoactivated PAGFP is stable, and negligible fluorescence is added by additional expression of this protein, mito-PAGFP could be a tool to image and quantify mitochondria-specific autophagy (mitophagy)-induced mitochondrial degradation rates in living cells. Furthermore, recent reports indicate that mitochondria can be transferred between cells (Ahmad et al., 2014); local photoactivation of mito-PAGFP is likely to reveal this process with the resolution unachievable with “standard” fluorescence imaging techniques.

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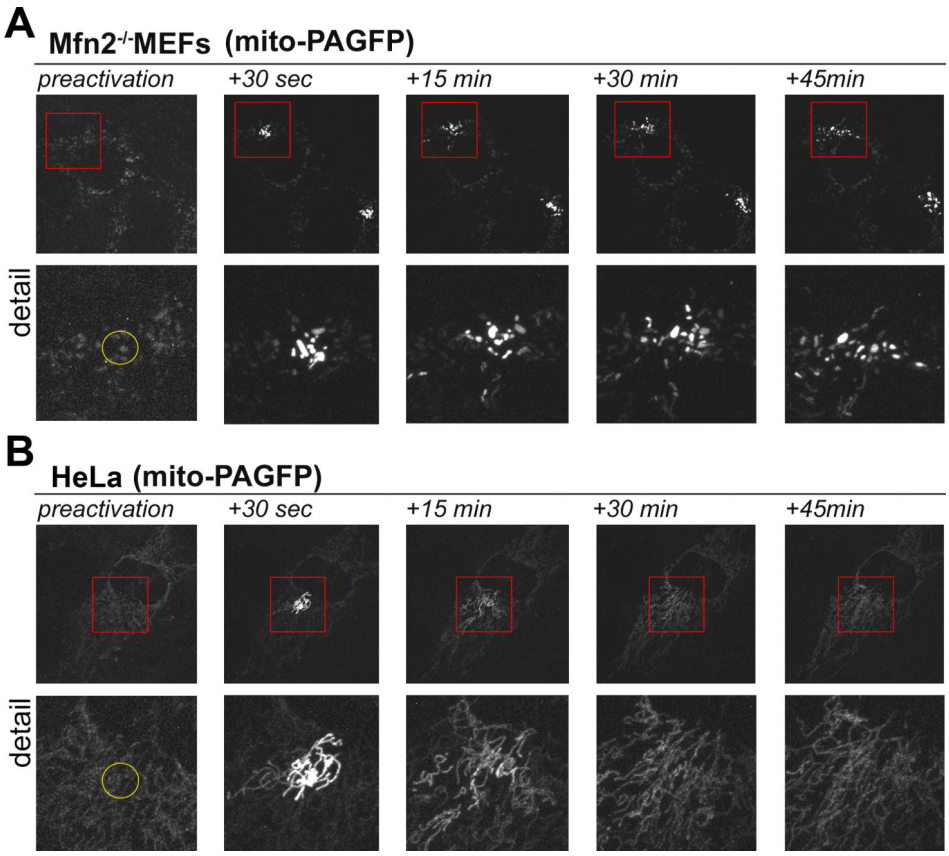

Figure 1. Imaging of mitochondrial fusion

Mitochondrial fusion deficient Mfn2$^{-/-}$ MEFs (A) and HeLa cells (B) were transfected with mito-PAGFP followed by confocal imaging (using 488-nm excitation light) of cells before (preactivation) and at various intervals, as indicated in the figure, after local ROI (yellow circle in detail images) was photoactivated with 405-nm light.