

Review

Mitochondrial fission and fusion dynamics: the long and short of it

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Maintenance of functional mitochondria requires fusion and fission of these dynamic organelles. The proteins that regulate mitochondrial dynamics are now associated with a broad range of cellular functions. Mitochondrial fission and fusion are often viewed as a finely tuned balance within cells, yet an integrated and quantitative understanding of how these processes interact with each other and with other mitochondrial and cellular processes is not well formulated. Direct visual observation of mitochondrial fission and fusion events, together with computational approaches promise to provide new insight.

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This article honors Richard Lockshin, whose vision saw so deeply and so clearly into the mechanisms of what he coined 'programmed cell death' even without all the technological advances in microscopy afforded to the work described here.

Mitochondrial Dynamics

Importance of mitochondrial fission and fusion. Mitochondrial fission and fusion are known to play roles in maintaining the (1) integrity of mitochondria, (2) electrical and biochemical connectivity, (3) turnover of mitochondria, and (4) segregation and protection of mitochondrial DNA (mtDNA).^{1–3} Moreover, the fission and fusion machinery have been implicated in programmed cell death pathways. The early fragmentation of mitochondria after an apoptotic stimulus is mediated in part by the mitochondrial fission proteins Drp1/DLP1/Dnm1 and Fis1, and blocking the actions of Drp1 with a dominant negative mutant inhibits the fragmentation of mitochondria, and in some cases also prevents the loss of mitochondrial membrane potential and release of cytochrome *c*.^{4–7} In addition, it was reported that the mitochondrial inner membrane fusion protein Opa1 is intimately involved in cristae remodeling and protection from cytochrome *c* release,^{8,9} suggesting that the fusion mechanisms are also important in normal mitochondrial cristae maintenance. For reasons that remain unclear, the maintenance of mtDNA cannot take place without mitochondrial fusion,¹⁰ and fusion of mitochondria has been directly implicated in preventing the accumulation of damaged mtDNA.^{11,12}

In the special case of neurons, mitochondrial dynamics appear to play an even more critical role, perhaps because neurons have unusually long processes, electrical properties, complex energy requirements and synaptic functions such as neurotransmitter release. For example, the mitochondrial fission machinery is intimately involved in the formation and function of synapses in dendritic spines and axons.^{13,14} A powerful tool for revealing the importance of Drp1 (dynamine-related protein) in these processes is a dominant-negative mutant of the fission protein Drp1, such as Drp1^{K38A} with impaired GTPase activity. Exogenously expressed, Drp1^{K38A} causes a loss of mitochondria from dendritic spines and a reduction in synapse number and size, whereas enhanced mitochondrial fission increases synapse formation.^{13,14} Depletion of endogenous Drp1 has also been shown to prevent mitochondria from distributing to synapses and to cause synaptic dysfunction^{15,14} consistent with a recently described human mutation in Drp1/DLP1 associated with severe abnormal brain development.¹⁶ Similarly, mutations in mitochondrial fusion factors cause a number of different human neuropathies^{17–19} and defective mitochondrial dynamics are increasingly implicated in many other neurodegenerative diseases.^{20,21}

What does Mitochondrial Morphology Tell about Fusion and Fission?

The fluidity and dynamic properties of intramitochondrial membranes, especially mitochondrial cristae, were first

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Abbreviations: Bcl-2, B-cell lymphoma/leukemia protein 2; CED-9, cell death-defective Bcl-2 homolog of worms; Drp1*, dynamine-related protein 1; FRAP**, fluorescence recovery after photobleaching; Fzo1, fuzzy onion protein 1 of mammals/yeast (*Drosophila* fuzzy onions); GFP, green fluorescent protein; Mfn1, mitofusin protein 1 (mammalian Fzo1); mt, mitochondrial (e.g. mtDNA, mtGFP, mtRFP); RFP, red fluorescent protein; TEM**, transmission electron microscopy

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suggested by transmission electron microscopy (TEM), and this technique has continued to reveal new insights into cristae remodeling.²² In addition, even before more advanced techniques, TEM and phase contrast microscopy revealed multiple mitochondrial morphologies, and dynamic changes in morphology and structure, suggesting direct division and fusion of individual mitochondrial organelles (reviewed in Bereiter-Hahn and Voth²³). Recent advancements in live cell imaging and high throughput strategies have rapidly advanced our appreciation for the importance of mitochondrial dynamics in cell death and survival; yet, relatively little is known about the molecular details, and much less is known about how these dynamic processes interface with mitochondrial energetics.

The *fzo* (fuzzy onions) gene is a fusion mediator that was first identified in *Drosophila* where loss-of-function mutations in *fzo* were shown to be responsible for defects in spermatogenesis.²⁴ The phenotype of these mutants is a failure of spermatid mitochondria to merge into two supersized organelles to form the Nebenkern that appears onion-like in cross section. The connected mitochondrial morphology of *fzo* mutant fly spermatids implied a defect in mitochondrial fusion, which was born out by elegant genetic experiments in yeast. Interestingly, loss-of-function mutations in the *Drosophila* homolog of human parkin, which causes autosomal recessive juvenile Parkinson disease when mutated, results in defective mitochondrial morphogenesis in the fly such that only one of the two giant mitochondria from the Nebenkern unfolds, leading to additional abnormal morphologies and condensation.²⁵

Genetic studies in the budding yeast *Saccharomyces cerevisiae* have identified over 20 genes that are required for normal mitochondrial morphology (Figure 1), and orthologs of some of these factors were subsequently identified in mammals (reviewed in Okamoto and Shaw¹ and Hoppins²⁶). The conclusion that yeast Dnm1 (ortholog of mammalian

Drp1) reduces the length of mitochondria by mediating organelle fission, rather than inhibiting fusion or other mechanism, is based on compelling genetic experiments using morphology-based readouts. Conversely, yeast Fzo1 (fuzzy onion-1), ortholog of mammalian mitofusin (Mfn1 and Mfn2), increases the connectivity of mitochondria by mediating fusion, rather than inhibiting fission. However, all of these conclusions were based only on indirect evidence rather than by directly enumerating fusion and fission events in live cells. The relatively recent advent of experimental methods that permit the direct observation of individual mitochondrial fission and fusion events are enabling more detailed analyses but are also introducing additional complications in quantifying and interpreting the data as explored in the following sections.

How do we know that Fzo/Mfn is a fusion protein rather than a fission inhibitor? A genetic approach in yeast provided indirect, yet, unambiguous evidence that yeast Fzo1 is a fusion protein.²⁷ During mating and zygote formation, the mitochondria from one haploid parent fuses to that of the other mating type parent. Therefore, to establish that yeast Fzo1 mediates fusion rather than suppressing fission, mitochondria of one haploid parent were stained with MitoTracker, while the other parent expressed mitochondrially targeted GFP (mitoGFP). One or both parents also carried a deletion or a temperature-sensitive mutation in the *FZO1* gene. At the permissive temperature, the merge of green and red mitochondria occurred normally, indicating that the mitochondria from one cell fused with that of the second cell. However, at the elevated temperature, where the mutant Fzo1 protein is non-functional or when the *FZO1* gene was deleted, which causes short punctate mitochondrial morphology, little or no merge of red and green mitochondria occurred following mating. Therefore, Fzo1 was required to facilitate mitochondrial fusion, and the presence of Fzo1 on one mitochondrion was not sufficient for it to fuse to a Fzo1-deficient mitochondrion.

Yeast proteins required for normal mitochondrial morphology

Protein	human homolog	Possible role/localization
Caf4	-	Mdv1-like, outer membrane fission, interacts with Dnm1, Mdv1 and Fis1
Cym1	-	Pitrilysin-like, intermembrane space metalloprotease
Dnm1	Drp1/DLP1	Dynamain-related GTPase, outer membrane fission
Fis1	Fis1	Outer membrane-anchored fission protein
Fzo1	Mfn1,2	Integral membrane protein with N-terminal GTPase
Gem1	-	Mitochondrial GTPase, EF-hand protein
Htd2	-	3-hydroxyacyl-thioester dehydratase
Mam3	-	Hemolysin-like
Mdm20	-	N-terminal acetyltransferase
Mdm30	-	F-box protein, mitochondrial fusion
Mdm31	-	Inner membrane
Mdm32	-	Similar to MDM31, inner membrane
Mdm33/She9	-	May promote mitochondrial inner membrane fission
Mdm34/Mmm2	-	Outer membrane
Mdm35	-	Intermembrane space
Mdm36	-	Unknown
Mdm37/PCp1	-	Rhomboid-like mitochondrial intermembrane protease
Mdm38/Mkh1	-	Mitochondrial inner membrane
Mdm39/Get1	-	Unknown
Mdv1	-	WD repeat protein, complexes with Dnm1 and Fis1
Mfb1	-	F-box protein
Mgm1	Opa1	Dynamain-like GTPase, cristae morphology, complexes with Fzo1 and Ugo1
Ugo1	-	Binds Fzo1 and Mgm1
Ups1	PREL1	Intermembrane space, regulates Mgm1

Figure 1 Yeast proteins required for normal mitochondrial morphology through their direct or indirect roles; for more information and citations, see *Saccharomyces* Genome Database at <http://www.yeastgenome.org/>

Furthermore, deletion of both *FZO1* and the mitochondrial fission gene *DNM1/Drp1* restored normal tubular mitochondrial morphology, but failed to restore mitochondrial fusion upon mating of these double knockouts.²⁸ Thus, the restoration of tubular morphology in the yeast double knockout presumably occurred through elongation of mitochondrial organelles rather than fusion. Subsequently, the mammalian counterparts, Mfn1 and Mfn2, were reported to mediate mitochondrial fusion, though this conclusion was also based on short mitochondrial morphology when the function of Mfn1/Mfn2 was impaired.^{29,30}

While these studies in yeast are compelling, it remains possible that the effects of mutation or deletion of *FZO1* on mitochondrial morphology are merely downstream consequences of a direct action on an entirely distinct process, such as energetics, ion flux, or other. However, additional evidence argues that Fzo/Mfn proteins are direct mediators of mitochondrial fusion. In particular, Fzo/Mfn proteins share significant amino-acid sequence similarity to the large dynamin GTPases known to be involved in membrane and vesicle fission.³¹ Furthermore, yeast Fzo1 protein was shown to be embedded in the mitochondrial outer membrane.^{10,27} More importantly, an *in vitro* assay was developed to demonstrate Fzo1-dependent fusion of mitochondria, thereby solidifying the role of Fzo/Mfn in membrane fusion.³² In these studies, yeast mitochondria labeled with mitochondrially targeted DsRed fluorescent protein were mixed with yeast mitochondria labeled with mitoGFP.³² Directly quantified mitochondrial fusion events were evaluated under different conditions, and a direct role for Fzo1 was confirmed. In addition, physiologic conditions, such as mitochondrial membrane potential and GTP, were required for mitochondrial fusion *in vitro*, and inner membrane fusion could be distinguished from outer membrane fusion.

Changes in morphology are generally taken as evidence for a change in the balance between fission and fusion.^{33–35} The use of morphology as a readout, is largely due to the difficulty of directly quantifying fission and fusion processes.

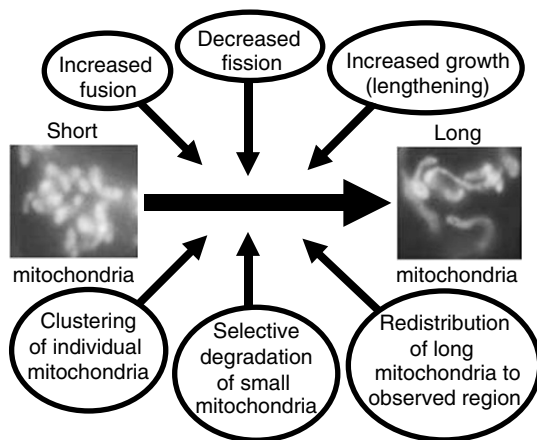


Figure 2 When the equilibrium between mitochondrial fission and fusion is reset to yield longer, more connected mitochondrial morphology, detected here in mammalian cells with mitoGFP, there are a number of potential mechanisms, or combinations of mechanisms to explain this altered morphology

Unfortunately, morphologic changes are not particularly revealing about biochemical processes. Other than a defective mitochondrial fission, fusion or growth extension and the countless potential sources of experimental bias, elongated/connected mitochondrial morphology could be due to changes in organelle distribution (or plane of focus), organelle stretching, or selective degradation of short mitochondria (Figure 2).

Indirect methods for assessing mitochondrial fission and fusion in mammalian cells

Cell–cell fusion. Modeled after the strategies employed in yeast, experimentally induced cell–cell fusion of two cell populations, labeled differentially with mitochondrially targeted RFP (mitoRFP) or mitoGFP, has been applied to monitor the mixing of mammalian mitochondrial matrices.^{36,37} These studies verified the conservation of fission and fusion machinery between yeast and mammals. As in yeast, mammalian mitochondrial fusion is also dependent on mitochondrial membrane potential and is not dependent on mitochondrial respiration, though some discrepancies were reported.³⁷

Fluorescence recovery after photobleaching (FRAP). To avoid potential artifacts from membrane-altering agents required to experimentally induce cell–cell fusion, new technologies were developed to more closely mimic *in vivo* conditions, in which physiologic manipulations and disease models can be studied. FRAP has been increasingly utilized to assess mitochondrial connectivity. Photobleached subcellular regions are monitored for refilling of ‘bleached’ areas with mitochondrially targeted fluorescent molecules from neighboring fluorescent (unbleached) areas to provide evidence that bleached mitochondria are physically connected to structures outside the bleached area. For example, this approach was used to show that the *C. elegans* Bcl-2 homolog, CED-9, promotes mitochondrial fusion in HeLa cells.³⁸

Photoactivatable mitoGFP. The ability to monitor fission and fusion of individual mitochondria was facilitated by photoactivatable mitoGFP. Photoactivatable GFP is a variant of *Aequora victoria* GFP, in which a single amino-acid substitution renders a stable, 100-fold increase in green fluorescence after laser activation.³⁹ Karbowski *et al.*⁴⁰ fused photoactivatable GFP to a mitochondrial targeting sequence. When coexpressed with a constitutively fluorescent marker, the interaction of activated and constitutively fluorescent mitochondrial populations can be monitored. For example, the loss of photoactivated fluorescence in a small region of mitochondria has been utilized as a measure of mitochondrial fusion, assuming that this loss of photoactivated fluorescence represents diffusion of fluorescence into a neighboring (presumably now fused) mitochondrion. This methodology was used to support a direct role for mitochondrial fusion by the Bcl-2 family proteins Bax and Bak.⁴¹ Photoactivated plus potentiometric mitochondrial fluorescence led to the interesting observation that one of the two daughter mitochondria produced by a fission event undergoes transient membrane depolarisation, a possible marker for mitochondrial deletion.⁴²

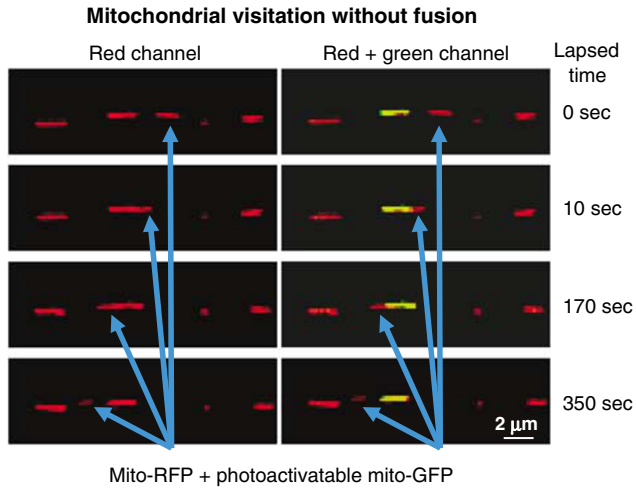


Figure 3 Monitoring mitochondrial movement. Time-lapse imaging was performed on a 14-day culture of primary rat cortical neurons cotransfected with mitochondrially targeted RFP and photoactivatable GFP; one mitochondrion was photoactivated (yellow). Blue arrows track the movement of an RFP-labeled mitochondrion past the photoactivated mitochondrion. Under RFP fluorescence detection alone, one cannot distinguish the monitored (red) and the photoactivated mitochondrion when both are juxtaposed, and it cannot be determined whether these mitochondria fuse. Using photoactivatable GFP, it can be concluded that these two mitochondria fail to fuse their matrices, even though they spend several minutes next to each other. Time 0 is at 8 min and 40 s from the start of a 15 min observation period

Towards a more Quantitative Understanding of Mitochondrial Fission/Fusion Dynamics

Mitochondrial fusion can be quantified using the above methods, when there is a relatively stationary mitochondrial population. However, recovery from photobleaching and dispersal of photoactivated fluorescence intensities will obviously be compromised where populations of mitochondria are more mobile. For example, rapid transport up and down neuronal axons and dendrites can move mitochondria considerable distances in seconds/minutes. Thus, increased 'recovery of fluorescence' after photobleaching could indicate organelle connectivity due to increased fusion, or it could reflect the appearance of a new mitochondrion moving into the bleached area; and conversely, 'loss of fluorescence' could result from diffusion into another mitochondrion upon fusion, or a fluorescently activated mitochondrion was transported out of the field. Consequently, these methods will be less useful for assessing mitochondrial fusion in healthy neuronal processes because of the motility and rapid transport of mitochondria along processes.

Direct visualization of photoactivated mitochondrial fluorescence into an adjacent mitochondrion has been described.⁴⁰ Using this method, together with improved image analyses, it will be possible to directly visualize, quantify, characterize, and manipulate mitochondrial fission/fusion dynamics in live neurons in real time. We have observed that selective photoactivation allows distinctions to be made between elongated mitochondria and structures that only appear continuous, but are instead clusters of individual mitochondria, or mitochondria that are being transported past each other (Figure 3). We believe that this methodology will

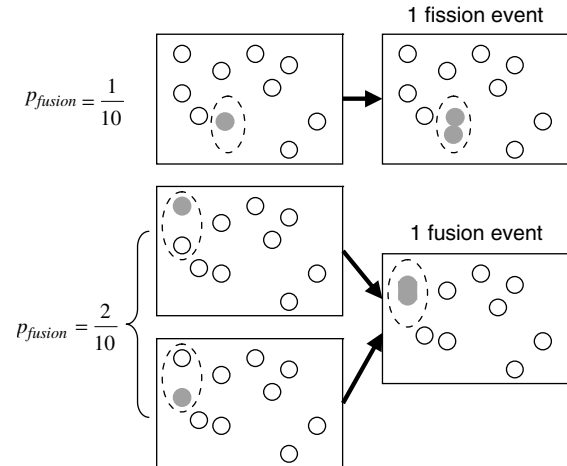


Figure 4 Quantifying mitochondrial fission and fusion. Consider a volume containing 10 mitochondria in which, on average, there is one fission event and one fusion event per a fixed time interval. If one of the 10 mitochondria is randomly selected for photoactivation, then one is twice as likely to activate a mitochondrion that will undergo a fusion event ($P_{fusion} = 2/10$) during the observation period, than one is likely to select a mitochondrion that will undergo a fission event ($P_{fission} = 1/10$). Thus, one will observe twice as many fusion events as fission events, even though the number of fission events equals the number of fusion events

ultimately allow direct quantification of mitochondrial fission, fusion, and transport in living cells to better characterize physiologic and disease parameters.

Future Directions

A systems biology approach for mitochondrial fission and fusion. Although photoactivatable mitoGFP allows one to characterize individual fission and fusion events in a population of mitochondria, there still remains the task of interpreting the data and connecting these measurements to falsifiable hypotheses about morphology. Measured fission and fusion rates are the observable consequences of complex interactions between individual mitochondria. At a very coarse level, observed fusion rates must certainly depend on how often mitochondria encounter each other, and on the likelihood that two mitochondria will fuse when they do encounter each other. Thus, observed rates of fission and fusion events will depend indirectly on parameters such as the number of mitochondria per cell and the mean velocity of the mitochondrial population. How can we tease these parameters apart to reach an understanding of the underlying fission and fusion processes? We believe that these questions will be answered by developing and testing specific mathematical models of mitochondrial fission, fusion, and morphology. Careful modeling will be required to transform experimental observations into quantitative statements about the balance between fission, fusion, and its impact on morphology.

A simple, but important, question highlights the need for careful modeling: how does one define (in a rigorous sense) the balance between fission and fusion? Experimentally, one can measure the ratio of fission and fusion events in GFP photoactivated mitochondria. How are these observed rates

connected to fission and fusion rates in the population? In the case of fission, if an observed fraction of GFP-activated mitochondria are observed to undergo fission within a specific time interval, the expected number of fission events in the population is obtained by multiplying the observed fission rate by the number of mitochondria in the volume. In the case of fusion, however, the situation is more subtle, as the same procedure (multiplying the observed fusion rate of photo-activated mitochondria by the number of mitochondria in the volume) will overestimate the number of fusion events in the volume by exactly a factor of two (Figure 4).

It becomes even more complex when one considers incorporating analysis of mitochondrial dynamics into steady-state physiological conditions. In other words, when subtle changes in mitochondrial fission or fusion occur inside cells, how do they affect and integrate with the balance of physiological parameters such as mitochondrial growth, biogenesis, degradation, metabolic function, and so on? If unregulated, one could predict that even small increases in the rates of mitochondrial fission would result in the eventual severe fragmentation of all mitochondria within a cell, but in normal *in vivo* steady-state conditions this does not occur, and changes in mitochondrial dynamics obviously integrate with the other mitochondrial processes. How is the balance maintained? A system-level understanding of how subtle regulation of mitochondrial fission and fusion interacts with the larger mitochondrial and cellular environment will clearly be critical for understanding the role of mitochondrial fission and fusion in physiologic cell function and dysfunction.

We often take for granted that there is a coordinated balance of mitochondrial fission and fusion that underlies not only morphology but also extends to involvement in cell death mechanisms and cell maintenance, and in neurons, to synaptic formation and function, and perhaps to neurodegeneration. However, tools to provide quantitative characterization of the complicated interrelationships between mitochondrial fission and fusion, and between mitochondrial dynamics and other cellular processes, have yet to be developed. As these are developed, we should be able to better incorporate scientific findings into physiologic implications for cell maintenance and disease.

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