Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP

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Twig, Gilad, Solomon A. Graf, Jakob D. Wikstrom, Hibo Mohamed, Sarah E. Haigh, Alvaro Elorza, Motti Deutsch, Naomi Zurgil, Nicole Reynolds, and Orian S. Shirihai. Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP. Am J Physiol Cell Physiol 291: C176–C184, 2006. First published February 15, 2006; doi:10.1152/ajpcell.00348.2005.—Assembling mitochondrial networks into networks supports fuel metabolism and calcium transport and is involved in the cellular response to apoptotic stimuli. A mitochondrial network is defined as a continuous matrix lumen whose boundaries limit molecular diffusion. Observation of individual networks has proven challenging in live cells that possess dense populations of mitochondria. Investigation into the electrical and morphological properties of mitochondrial networks has therefore not yielded consistent conclusions. In this study we used matrix-targeted, photoactivatable green fluorescent protein to tag single mitochondrial networks. This approach, coupled with real-time monitoring of mitochondrial membrane potential, permitted the examination of matrix lumen continuity and fusion and fission events over time. We found that adjacent and intertwined mitochondrial structures often represent a collection of distinct networks. We additionally found that all areas of a single network are invariably equipotential, suggesting that a heterogeneous pattern of membrane potential within a cell’s mitochondria represents differences between discrete networks. Interestingly, fission events frequently occurred without any gross morphological changes and particularly without fragmentation. These events, which are invisible under standard confocal microscopy, redefine the mitochondrial network boundaries and result in electrically disconnected daughter units.

Mitochondria conduct essential cell processes, including oxidative phosphorylation, Ca2+ signaling, and apoptosis (15, 18, 22). Central to these activities is communication between mitochondria within the cell, a process greatly influenced by mitochondrial morphology and dynamics. Mitochondria regularly undergo morphological changes in which they fuse, split, and translocate, resulting in the assembly of webs or in separation into small fragments (30).1

A number of studies have demonstrated the relevance of this form of dynamics for mitochondrial function. Electron microscopic (EM) studies have shown that juxtaposed mitochondria could form networks with a continuous matrix lumen (1, 11).2 Such networks are thought to enable sharing of diffusible nutrients and proteins, as well as electrochemical gradients (30). Pharmacological inhibition of network formation results in reduced mitochondrial oxygen consumption and fuel metabolism (5). Moreover, converting the mitochondrial networks to separate units (fragmentation) has been shown to impair Ca2+ wave propagation through the mitochondrial web (28) and alter the susceptibility of the cell to apoptosis (5, 16, 26). These studies emphasize the significance of network connectivity in mitochondrial function in health and disease. Further elucidation of the consequences of proper and failed mitochondrial communication requires improved understanding of the networks’ basic dynamics, electrophysiology, and biochemical players. Examination of the morphological and electrochemical properties of mitochondrial networks will establish the flexibility and limitations of mitochondrial signal propagation. Although the mitochondrial web remains the most commonly studied unit, the connectivity within the web, as would be determined by the occurrence, the dynamics, and the flexibility of networks within the single web, is still unclear.

Functional studies of mitochondrial membrane potential (Δψm) and mitochondrial calcium uptake have shown high levels of mitochondrial heterogeneity, even in the presence of web structures questioning connectivity and electrical coupling within the web (6, 7, 11). Asymmetric distribution of Δψm along a single tubular mitochondrial structure was demonstrated with JC-1 (4, 25) and tetramethylrhodamine methyl (TMRM) ester perchorlate staining (9). These studies observed voltage differences 15 mV along “a single mitochondrion.” On the other hand, time-lapse measurements from mitochondria in COS-7 cells and myocytes demonstrated that mitochondria within a web in these cells display synchronized electrical activity and are therefore presumed to be electrically coupled (8).

The inconsistency of these findings emphasizes the importance of accurately defining mitochondrial networks. To do so, a methodology is required that accounts for 1) the inherent morphological complexity of mitochondrial structures, 2) the continuous movement of mitochondria, and 3) fusion and fission events that continuously remodel the network.

1 Mitochondrial web: Any organization of mitochondria that is not fragmented or interrupted regardless of lumen continuity or electrical coupling. Common forms are ramified tubules and clusters.

2 Mitochondrial network: Any group of mitochondria or mitochondrial architectures that share a continuous matrix lumen. Alternative terms are “single mitochondrion” and “mitochondrial unit.”

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In this study, we selectively labeled individual mitochondrial networks within the intact living cell, using mitochondrial matrix-targeted photoactivatable green fluorescent protein (PA-GFPmt). This methodology allowed extended observation and subsequent integration of mitochondrial structures, matrix lumen continuity, ΔΨm coupling along a mitochondrial structure, and mitochondrial fusion/fission events. We found that 1) proximity between two points along a complex mitochondrial web poorly predicts whether these two points share a continuous lumen; 2) any two points along a mitochondrial network that are luminally continuous are invariably equipotential; and 3) electrical dissociation occurs regularly along a previously connected network and appears to be accompanied by luminal discontinuity. Remarkably, these events frequently leave the morphology of the mitochondrial network unchanged, highlighting the subtlety of relationships between mitochondrial networking and morphology.

**MATERIALS AND METHODS**

*Cell culture and transfection.* COS-7 and K562 cells were cultured in DMEM without phenol red (Invitrogen, Eugene, OR) supplemented with 10% standard fetal bovine serum (HyClone, Logan, UT), 1% penicillin-streptomycin, and 2 mM l-glutamine (Invitrogen) at 37°C in 5% CO2-95% air atmosphere. INS-1 cells (clone 823-13) were cultured in RPMI 1640 containing 10% standard fetal bovine serum, 1% penicillin-streptomycin, 2 mM l-glutamine, 1 μl of 2-mercapto-ethanol, 5 mM NaHCO3, 2 mM HEPES, 2 mM pyruvic acid, and 11 mM glucose (Invitrogen). Transient transfections of COS-7 and INS-1 cells were performed with FuGene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Cells were ~80% confluent at transfection. Transfection efficiency was 75% in COS-7 cells and 10% in INS-1 cells.

*DNA plasmids and constructs.* DNA plasmid containing PA-GFP was a gift from Dr. Lippincott-Schwartz (NICHD). DNA coding for the mitochondrial targeting sequence of COX VIII was amplified by PCR and inserted immediately 5' to PA-GFP, thus directing it to the mitochondrial matrix as PA-GFPmt. The new plasmid was validated by sequence analysis at the Tufts University core facility (Boston, MA). ABCB10-PAGFP was constructed as described in Reference 12.

*Confocal microscopy.* Mitochondria were labeled with the mitochondrion-specific dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Invitrogen). Freshly prepared TMRE was added to culture in DMSO to give a final concentration of 7 nM and incubated for 45 min before imaging. Cells were kept in the dark to minimize phototoxicity and maintained at 37°C. Confocal microscopy was performed on live cells in glass slide-bottomed dishes (MatTek, Ashland, MA) with a Zeiss LSM 510 Meta microscope with a plan apochromat ×100 (numerical aperture = 1.4) oil immersion objective. COS-7 and INS-1 cells were transfected with PA-GFPmt and allowed to express and accumulate the protein in the mitochondrial matrix for 48 h. A transition to its active (fluorescent) form was achieved by photoisomerization with a two-photon laser (750 nm) to give a 375-nm photon equivalence at the focal plane. This allowed for selective activation of regions that are <0.5 μm2. In the absence of photoactivation, PA-GFPmt protein molecules remained stable in their preactivated form. The presence of preactivated PA-GFPmt was detected with high-intensity excitation at 488 nm (25-mW laser set at 1%) in combination with a fully opened pinhole.

With the multitrack scanning mode of the LSM-510 microscope, red-emitting TMRE was excited with a 1-mW, 543-nm helium/neon laser set at 0.3%, and emission was recorded through a BP 650- to 710-nm filter. Activated PA-GFPmt protein was excited with a 25-mW, 488-nm argon laser set at 0.2%. Emission was recorded through a BP 500- to 550-nm filter.

*Imaging of nonadherent cells.* To perform confocal microscopy on suspension cells we used LiveCell Array technology (Molecular Cytomics, Ramat Gan, Israel). The array prevents the nonadherent cells from moving on the slide, thus enabling imaging at high resolution. The LiveCell Array is a densely packed array of 20-μm hexagon wells, each designed to contain an individual living cell in culture medium (27, 31).

*Mitochondrial membrane potential analysis.* Because changes in TMRE fluorescence intensity (FI) can originate from either changes in ΔΨm or mitochondrial movement in and out of the focal plane, we used the ratio in FI of TMRE and activated PA-GFPmt. The rationale for using this approach arises from the similar effect that mitochondrial movement has on both TMRE and PA-GFPmt FI that thereby leaves the TMRE-to-PA-GFPmt ratio largely independent of the exact focal plane. However, because the FI of the PA-GFPmt molecule is independent of ΔΨm (see Results and Fig. 1), the TMRE-to-PA-GFPmt ratio reflects changes in ΔΨm.

*Image processing and thresholding.* Image processing was assisted by MetaMorph software (Molecular Devices). The algorithm developed for these experiments extracted structures that were green and that were larger than 10 pixels with the “Integrated Morphometry Analysis” function. These areas were interpreted to be mitochondria, and their TMRE FI was also recorded. This procedure enabled the selection of mitochondrial structure by using very low threshold in the green channel (~10% of the image average intensity). To set the level of thresholding in the green channel, a test threshold function measured the average intensity of the mitochondrial. A value equal to two-thirds of the average intensity was then used as the lower threshold (inclusive threshold). This algorithm ensures that >90% of the mitochondrial pixels will be used for measurements. Before analysis, all images were scanned to verify that intensity values were at all times below saturation level and therefore the upper threshold was not applied. To verify that GFP thresholding does not impose an artifact of cutting the mitochondrial objects short, we followed events in which mitochondrial GFP became diluted because of fusion between mitochondria and observed the expected expansion of the object (see Supplemental Fig. S2).3 As the mitochondrial areas were defined by the green pixels (GFP), there was no need to apply threshold in the red channel (TMRE).

*Image analysis.* Dividing the red and green images yielded a third ratio image. This procedure was performed for every time point for each of the three channels: PA-GFPmt, TMRE, and ratio of PA-GFPmt to TMRE of the individual mitochondrion. Ratio image data were then used to calculate the change in ΔΨm. This analysis was performed for the entire mitochondria or areas therein. Images from the green channel time series were used to follow the change in mitochondrial shape and size.

*Calculation of membrane potential.* As described by O’Reilly et al. (20), the ΔΨm-dependent component of TMRE will accumulate in a Nernstian fashion proportional to the intensity of its fluorescence. The non-ΔΨm-dependent component of TMRE, also known as the binding component, can be ignored as it is fixed and voltage independent (20). A change in mitochondrial membrane potential can thus be calculated by employing the equation

\[
\Delta \Psi_m = 61.5 \times \log \left( \frac{F_{PAGFP_{mt}}}{F_{TMRE_{A}}} \right)
\]

at 37°C, where \( F_{TMRE_{A}} \) and \( F_{TMRE_{B}} \) represent the fluorescence intensities of TMRE at two time points and \( F_{PAGFP_{mt}} \) and \( F_{PAGFP_{mt,B}} \) represent the fluorescence intensities of PA-GFPmt. This formula is based on the Nernst equation and is a modified version for ion concentrations across a potentiated membrane used in experi-

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3 Supplemental data for this article is available online at [http://ajpcell.physiology.org/](http://ajpcell.physiology.org/).
ments that included the sole use of TMRE (20). Specifically, the \( \text{(FI}_{\text{PA-GFP}_{m}}/\text{FI}_{\text{PA-GFP}_{m}}) \) term normalizes the FI of TMRE to fluctuations in FI of PA-GFP\(_{mt} \), thus controlling for shifts in focal plane, volume changes, and other non-\( \Delta \psi_{\text{m}} \)-dependent parameters that potentially affect the FI of TMRE.

**RESULTS**

**Defining the boundaries of a mitochondrial network.** The decomposition of the mitochondrial web into its discrete units requires continuous real-time imaging of the mitochondrial matrix because of fusion and fission events that continuously change the size and shape of a mitochondrion (14). In the present study, such decomposition was achieved by double labeling mitochondria with TMRE and PA-GFP\(_{mt} \). TMRE, a mitochondrial voltage-sensitive dye, labeled all mitochondria indiscriminately to reveal the gross morphology of the mitochondrial architecture. In parallel, PA-GFP\(_{mt} \) was transiently expressed by transfection. Before photoactivation of PA-GFP\(_{mt} \), mitochondria emit only the red TMRE fluorescence, as shown here for a COS-7 cell and an INS-1 cell (whole cell images in Figs. 1A and 2A, respectively). During photoactivation of a distinct branch of the mitochondrial web, the PA-GFP\(_{mt} \) molecules in that region irreversibly isomerize to their active, fluorescent state. The diffusion boundaries of each mitochondrial network tested were revealed when the isomerized PA-GFP was equilibrated across the lumen, a process that took 1–5 s. The network boundaries were therefore defined by the continuity of its matrix space, within the larger, TMRE-stained mitochondrial web. Interestingly, the nonfragmented shape of the web as revealed by TMRE alone does not reliably predict the boundaries of a network (Figs. 1A and 2A; see Fig. 4A). This phenomenon was observed in all cell types examined, including INS-1 (n = 37), COS-7 (n = 23), and K562 (n = 10) cells.
Controlling for laser phototoxicity. Careful attention was given to the possibility of detrimental cellular effects introduced by the experimental methodology. We defined the time and intensity limits of laser exposure that initiated changes in ∆Ψm or mitochondrial morphology in cells treated with TMRE and conducted our experiments well below these thresholds (unless stated otherwise). In COS-7 and INS-1 cells, two-photon excitation was executed over a wide range of dosages (see Supplemental Fig. S1A). We found that excitation for 600 ms/μm² at 1-mW laser intensity at the objective is the threshold dosage for both cell lines, above which a reduction in ∆Ψm can be observed. Doses less than this had no effect on ∆Ψm. All subsequent experiments using two-photon illumination were conducted with a duration of 150 ms/μm² and an intensity of 1 mW, unless stated otherwise. Two additional control experiments are described in the supplemental data for this article (see Laser- and fluorophore-induced toxicity and Fig. S1B).

Diffusion properties of mitochondrial GFP. Three-dimensional EM tomography studies have suggested the possibility that the diffusion rates of proteins and even ions are significantly attenuated by the density of matrix cristae (10). To account for this possibility, we compared the diffusion boundaries of matrix as well as inner membrane activated PA-GFPᵐᵗ to the boundaries determined by electrical coupling. Two types of experiments were conducted to validate the reliability of PA-GFP in defining the network lumen boundaries, both ruling out the possibility that matrix structures permit electrical coupling but block protein diffusion (Fig. 1, A and B, and Fig. 2A). In COS-7 cells expressing matrix PA-GFPᵐᵗ, individual branches of the web of mitochondria were targeted with a two-photon laser at a phototoxic dose (Fig. 1A, top). Ten seconds after photoactivation the structure of the network matrix was revealed by the spread of PA-GFPᵐᵗ molecules (Fig. 1A, middle). One minute after the application of the high-dose laser, the mitochondrial membrane collapsed and TMRE fluorescence disappeared from the electrically coupled segment (Fig. 1A, top). The electrically coupled segment and the area defined by GFP diffusion assay were colocalized in COS-7 cells (n = 15), in INS-1 cells (n = 10, see example in Fig. 2A), and in nonadherent human erythroleukemic K562 cells (n = 5). Compared with matrix PA-GFP, when PA-GFP was fused to an inner membrane protein (ABCB10), diffusion was slow (Fig. 1B and Supplemental Fig. S3). The time it took diffusion to reach steady state was 60–300 s for ABCB10-PAGFP (n = 30), compared with 1–5 s (n = 300) for matrix GFP. Although slower, diffusion of ABCB10-PAGFP resulted in complete filling of mitochondrial segments that were electrically coupled to the spot of photoactivation. Electrically coupled regions were identified with phototoxic-range laser dosages during the photoactivation step. Through lateral diffusion ABCB10-PAGFP covered the depolarized segments and did not proceed to neighboring polarized mitochondria even when these were adherent to the tested mitochondrial branch (Supplemental Fig. S3; n = 10).

Experiments described in Fig. 2A and in Supplemental Fig. S2 control for the possible short-cutting artifacts that may be introduced by thresholding in the GFP channel. Figure 2A demonstrates the ability of PA-GFPᵐᵗ to diffuse across elongated mitochondrial networks and completely cover segments that are electrically coupled. Supplemental Fig. S2 shows that although GFP becomes diluted because of diffusion across the
network, its detection is not limited by possible thresholding artifacts. Mitochondrial membrane potential of individual mitochondria. The dual labeling of a mitochondrion with TMRE and PA-GFPmt was used to monitor $\Delta \psi_m$ of the individual mitochondrial network in real time. TMRE FI responds to $\Delta \psi_m$, whereas PA-GFPmt FI does not. This is demonstrated by the stability of PA-GFPmt FI after depolarization that was induced either by laser overdose (Fig. 1A) or by the addition of FCCP, a mitochondrial uncoupler (Fig. 1C). Because TMRE and PA-GFPmt FIs are similarly influenced by changes in focal plane, the ratio product, TMRE/GFP, retains the voltage dependence of TMRE but is independent of the exact focal plane. Figure 1D demonstrates this effect in a mitochondrion that was studied with nine sections along its z-axis (distance interval of 0.3 μm). TMRE FI is strongly affected by the amount of the mitochondrion’s area captured at each of the focal plains; the highest values are found at focal planes that capture the largest area of the mitochondrion. Thus a drop in the mitochondrion’s area captured at a certain focal plane can manifest as an artificial drop in $\Delta \psi_m$ at TMRE FI. This phenomenon can be overcome by using the ratio of TMRE and PA-GFPmt FIs, as demonstrated by the stable ratio values in sections where the mitochondrion could be visualized. Figure 1E summarizes results of 10 similar experiments, demonstrating that even when the area of the mitochondrion captured at a specific focal plane drops to 30% of the mitochondrial area, the TMRE-to-GFP ratio is unaffected, in contrast to the TMRE FI. When the area of a given mitochondrion dropped to <20% of its maximal area, the TMRE-to-GFP ratio signal became unreliable.

Synchronized electrical activity over time is a fundamental property of electrical coupling. To test whether networks identified by PA-GFPmt diffusion are also synchronized, we monitored $\Delta \psi_m$ from multiple locations in individual networks. An example of such an experiment is shown in Fig. 2A. TMRE/GFP was measured from four regions of interest (ROIs 1–4) in a network from which changes in $\Delta \psi_m$ were calculated (see MATERIALS AND METHODS) and graphed over time. The calculated $\Delta \psi_m$ in all ROIs were synchronized over time, implying that the four loci were electrically coupled. For comparison, the change in $\Delta \psi_m$ (based on the change in TMRE fluorescence only) was also measured from two additional ROIs that were not photoactivated and were located on the outside margins of the GFP image (ROIs 5 and 6). It should be noted that the depolarization in ROIs 1–4 was absent in ROIs 5 and 6 (3.75-min images in Fig. 2A).

Electrical coupling and dissociation along the mitochondrial network. Whereas the test for luminal continuity determines the boundaries of a certain network at the time of photoactivation, electrical coupling can be continuously monitored and report on possible dissociations that might occur with time. To follow the connectivity dynamics of the individual network, we used the PA-GFPmt approach to first identify and label the boundaries of each network and then follow its connectivity by monitoring electrical coupling and synchronicity within the network. For each experiment, the image of the mitochondrial network (identified by the photolabeling of PA-GFPmt) was divided into two to seven regions, and the TMRE-to-GFP ratio was measured and $\Delta \psi_m$ was derived for each region. Although electrical coupling was found in the first 30 s in all networks tested (maximal difference within the network was $1.13 \pm 1.45$ mV; $n = 20$), some networks demonstrated electrical dissociation at later time points. An example of a network that developed electrical dissociation is shown in Figs. 2, C and D. It should be noted that during the 1.75-min time interval after photoactivation all four ROIs were equipotential, but subsequently $\Delta \psi_m$ measured from ROIs 1 and 2 diverged from those of ROIs 3 and 4 (arrow in Fig. 2D). This dissociation in $\Delta \psi_m$ occurred without any apparent gross change in the inner membrane morphology or location of the mitochondrion, as judged from the TMRE image (Fig. 2C). The voltage difference between ROIs 1 and 2 and ROIs 3 and 4 ranged between 5 and 12 mV, indicating an increase in electrical resistance and dissociation between ROIs 2 and 3 (Fig. 2D). After an additional 2 min, the electrically distinct loci reconverged for 1 min and then dissociated again for the rest of the measurement.

To quantify the frequency of intranetwork electrical dissociation we tracked $\Delta \psi_m$ at multiple locations of each COS-7 network tested for a period of 20 min. This analysis considered events according to the following criteria: 1) the networks were longer than 4 μm, ensuring appropriate spatial resolution between the ROIs, and 2) splitting of the network into separated fragments did not occur during the experiment. Diffusion boundaries for all networks were first defined by photoactivation of PA-GFPmt as above. In 11 networks studied, we identified a total of 10 electrical dissociation events (voltage gradient $>4$ mV). Figure 2E shows the cumulative probability for an intranetwork electrical dissociation over time. The cumulative probability for a silent fission event was calculated as the total number of dissociation events over a given time interval divided by the total number of experiments. Linear fit to the curve shows that the cumulative probability for intranetwork electrical dissociation is dependent on time, with a probability of 50% after $\sim$5 min of tracking.

We hypothesized that electrical dissociation is a consequence of fission and, as such, should be accompanied by a block in matrix continuity that limits the diffusion of PA-GFPmt. To test for this, two-stage photoactivation experiments were conducted. The first photoactivation step determined lumen continuity and enabled the monitoring of the potential difference between different loci within boundaries of each network tested (Figs. 1 and 2). Once a potential difference was detected between two loci, a second photoactivation was applied to one of these loci and the PA-GFPmt FI was determined. It is expected that in the case of a block in lumen continuity between the two sections, the concentration of PA-GFPmt molecules will selectively increase in the section where the second photoactivation was applied while the other section will remain unchanged. However, if lumen continuity holds, PA-GFPmt fluorescence in both sections would be expected to increase equally. Figure 3 shows an example of such an experiment in a COS-7 cell. After initial photoactivation of the tip of the structure, an elongated network was revealed (Fig. 3A), as judged by the homogeneous distribution of the activated PA-GFPmt and the similar membrane potential values, measured at ROIs 1 and 2 (event 1 in Fig. 3C). The similarity in the membrane potential held for $\sim$4.5 min, at which point the two loci diverged to a voltage separation of $\sim$5 mV. After this divergence, a second photoactivation was applied to the area of ROI 2 to determine the degree of lumen continuity between ROIs 1 and 2. The PA-GFPmt distribution following
the second photoactivation revealed a restricted distribution of the PA-GFP

continuity accompanies the electrical dissociation between two points along the mitochondrial network, even in the absence of gross morphological changes in web architecture.

Walking on the web: assessing the size of mitochondrial networks. These results suggest that the functional network, defined by lumen continuity, is only a fraction of the mitochondrial web that is defined by adherence. We sought to quantify this fraction for a sample population of mitochondria. After each photoactivation the area of the newly illuminated network was measured, and its relative fraction of the total area of mitochondria in the cell was calculated. This normalization procedure compensated for the variability in size and shape of the mitochondrial network between cells and within the same cell. Analysis of the size of 127 networks from 10 different cells identified three peaks at 0.8%, 2.0% and 4.2% of total mitochondrial area. Overall, the normalized area of the majority of the mitochondria analyzed was <1% of the total mitochondrial web. Similar results were found in INS-1 cells and in K562 cells, which are nonadherent leukemic cells (data not shown).

DISCUSSION

Fragmentation or assembly of the mitochondrial web has been the focus of numerous studies, demonstrating the functional significance of mitochondrial architecture (2, 16, 26, 28). In this study we examined the organization and dynamics of mitochondrial networking within the intact mitochondrial web. In the three types of cells tested in this study, the mitochondrial web is composed of numerous networks. Each network of mitochondria, defined by luminal continuity, is invariably equipotential, and its boundaries cannot be predicted without consideration of these parameters. Separation of the network occurs frequently and often without any resultant morphological change. The morphological and biophysical tests employed here may prove useful for future studies attempting to precisely elucidate electrophysiological behavior of single mitochondria in the live cell environment.

The technique: simultaneous imaging of TMRE and PA-GFP

Mitochondrial matrix-targeted PA-GFP <sub>mt</sub> was used in previous studies to identify and quantify fusion events by observing the diffusion of GFP through the mitochondrial matrix (14). The present work establishes three additional applications of PA-GFP<sub>mt</sub> photoactivation: 1) identification of network boundaries in a complex mitochondrial architecture, 2) the use of PA-GFP<sub>mt</sub> with TMRE to derive a ratiometric value for ΔΨ<sub>mt</sub>, and 3) the use of the PA-GFP<sub>mt</sub> image as a quality control index to identify and exclude images from which an accurate ratio cannot be deduced because of artifacts arising from movement of mitochondria across focal plains. Such measurements would not be possible if diffusion of PA-GFP<sub>mt</sub> throughout the matrix was restricted or delayed. This observation is in agreement with previous studies that measured the high diffusion rate of GFP in the mitochondrial matrix (21). The ratio quotient of the FIs of TMRE and PA-GFP<sub>mt</sub> is used to quantify small changes in ΔΨ<sub>mt</sub> independent of focal plane shift. Limiting scanning to a single plane maximizes time resolution and reduces laser-induced damage.

Several limitations, however, must be taken into account when using this approach. Mitochondrial dyes have been shown to modify mitochondrial function by inhibiting respira-
tery chain activity and by generating oxidative damage when absorbing the laser photons (19). TMRE is the optimal probe for our studies because the tetramethylrhodamine group of mitochondrial dyes carries relatively low toxicity and TMRE can be applied at concentrations well below those that inhibit respiratory function (23). This group of dyes is also preferable for visualizing rapid changes in $\Delta \psi_m$. The by-products of the interaction between the laser and TMRE, besides depending on the laser intensity and duration of exposure, reflect the intracellular concentration of the dye, the cell type, and the ingredients of the medium. Therefore, laser dosages must be tested for phototoxicity for every cell, dye concentration, and medium used. The imaging of activated PA-GFP<sub>mt</sub> with a 488-nm laser carries additional toxicity potential. The 488-nm laser delivers higher energy that is also absorbed by TMRE. To enable the detection of PA-GFP<sub>mt</sub> with a low-intensity 488-nm beam, it is essential to have high density of activated PA-GFP<sub>mt</sub> in the target mitochondrion. This can be achieved by high-dose photoactivation with the two-photon laser (which may potentially damage the organelle) or by high levels of expression of the PA-GFP<sub>mt</sub> transgene with a strong promoter and Kozak sequence. Together, these considerations might limit the cell types that can be used for these studies because of expression efficiency and/or sensitivity to laser excitation.

**Longitudinal voltage profile of single mitochondrial unit.**

Our results indicate that an individual network with a continuous lumen is invariably equipotential (Figs. 1–3). However, although the data shown here demonstrate that adherent networks that do not share a continuous lumen are frequently not electrically synchronized (Figs. 1–3), previous works report that neighboring mitochondria can synchronize (1, 3, 17, 24). The mechanism behind this phenomenon has been described in cardiomyocytes and skeletal muscle as high-conductance intermitochondrial junctions of which the structural and molecular component have not yet been resolved. Synchronicity alone is therefore not necessarily indicative of luminal continuity; similarly, disynchronicity of two adherent mitochondria by itself is not an indication of fission unless the two were found to have a continuous lumen before.

Previous studies addressing the voltage profile along the individual mitochondrion have reached conflicting conclusions. Laser photobleaching of a narrow area in the mitochondrial network led to extensive discharge of the mitochondrial dye throughout the individual network, suggesting equipotential distribution of $\Delta \psi_m$ (1). These results were challenged by imaging of $\Delta \psi_m$ without intervention using confocal microscopy and the membrane potential dye JC-1, suggesting that individual mitochondria (defined by visual inspection of confocal images) frequently have large voltage gradients (4, 9, 25). Although the interpretation of JC-1 images has been questioned, such a voltage gradient may be compatible with a dense architecture of the mitochondrial cristae, which was suggested to generate high-resistance narrowing within the matrix lumen (10, 11). If indeed possible, such narrowing will create a functional fission (no lumen continuity or electrical coupling) but with continuous inner membranes. We suggest that this scenario is very unlikely for the following reasons. Studies in isolated mitochondria showed that GFP diffusion paralleled inner membrane fusion, indicating that diffusion boundaries should parallel membrane discontinuity and, thus, fission. In addition, we show here that inner membrane proteins do show lateral diffusion in the inner membrane and that this diffusion is restricted to regions that are electrically coupled, again suggesting that there is no membrane continuity between segments that are not electrically coupled.

Fig. 4. Average size of an individual network. A: image of a COS-7 cell before photoactivation. Cell is stained with TMRE. Calibration bar, 20 μm. B: images of the mitochondrial web before and after 1 and 2 photoactivation steps (1–3, respectively). Photoactivation regions are indicated by dashed squares. Calibration bar, 2 μm. C: average area of a single network (normalized to the total area of the mitochondrial web of that section). Data include 127 photoactivation events in 12 experiments.
The present investigation approaches the mitochondrial voltage profile question with a method that avoids laser photo-bleaching and continuously quantifies $\Delta \phi_{in}$ and assesses luminal continuity. The latter consideration, to our knowledge, has not been taken into account in previous works and is critical, given the remarkable frequency of fusion and fission events that continuously change the mitochondrial boundaries. We provide evidence to show that the mitochondrial network is invariably equipotential (Fig. 1) and suggest the definition of a “single mitochondrion” as a source for the conflicting results. Microscopic inspection using mitochondrial membrane probes alone allows a string of juxtaposed but disconnected mitochondria to appear identical to a network of elongated mitochondria with continuous matrix.

Morphologically silent fissions along the mitochondrial network. Insights into a possible mechanism of silent fission can be found in previous reports. A possible scenario for silent fission would be an event in which the inner membrane undergoes fission while the outer membrane remains intact. This will result in electrical dissociation with lumen division but no organelle fragmentation. EM studies indeed demonstrate the feasibility of such phenomena, showing that the matrix can be found segmented within a tubule of continuous outer membrane (29). Jakobs et al. (13) showed intermittent separation of the mitochondrial matrix within intact outer membrane in living yeast cells, but it is unclear whether these separated matrix volumes were generated by fissions of initially continuous matrices or whether they represent a static structure of outer membranes containing fragmented matrixes (13). Such structures were observed as an intermediate stage of fusion in isolated mitochondria (17) and more recently during pharmacological inhibition of glycolysis and through the dissipation of inner membrane potential (16a).

Here we provide evidence that fission of the mitochondrial inner membrane with concomitant matrix division can occur in the absence of mitochondrial fragmentation, indicating that fission and fragmentation are two distinct processes.

The intermittent disconnections elicited by silent fissions are consistent with the observation that a single mitochondrial network constitutes only a 1–2% fraction of the entire elongated mitochondrial webs, with occasional networks having a doubled or tripled size (Fig. 4). The molecular mechanism and the functional significance of silent fissions require elucidation. Our results suggest that silent fissions may prove to be at least as common as visible fragmentation events. Given the profound influence of networking on mitochondrial function, silent fissions may act as an important modulator of cell metabolism in health and disease.

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