Analysis of Mitochondrial Dynamics and Functions Using Imaging Approaches

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ABSTRACT

Mitochondria are organelles that have been primarily known as the powerhouse of the cell. However, recent advances in the field have revealed that mitochondria are also involved in many other cellular activities like lipid modifications, redox balance, calcium balance, and even controlled cell death. These multifunctional organelles are motile and highly dynamic in shapes and forms; the dynamism is brought about by the mitochondria's ability to undergo fission and fusion with each other. Therefore, it is very important to be able to image mitochondrial shape changes to relate to the variety of cellular functions these organelles have to accomplish. The protocols described here will enable researchers to perform steady-state and time-lapse imaging of mitochondria in live cells by using confocal microscopy. High-resolution three-dimensional imaging of mitochondria will not only be helpful in understanding mitochondrial structure in detail but it also could be used to analyze their structural relationships with other organelles in the cell. FRAP (fluorescence recovery after photobleaching) studies can be performed to understand mitochondrial dynamics or dynamics of any mitochondrial molecule within the organelle. The microirradiation assay can be performed to study functional continuity between mitochondria. A protocol for measuring mitochondrial potential has also been included in this unit. In conclusion, the protocols described here will aid the understanding of mitochondrial structure-function relationship. *Curr. Protoc. Cell Biol.* 46:4.25.1-4.25.21.

Keywords: mitochondria • imaging • live cell

INTRODUCTION

Mitochondria are double membrane-bound organelles harboring their own DNA; they have been classically thought to be the powerhouse of the cell. Recent studies of mitochondria in the cellular context have revealed several other functional contributions of the organelles, including redox homeostasis, lipid modification, calcium homeostasis, and cell death processes (Scheffler, 2001). The inner membrane of mitochondria maintains a transmembrane gradient of ions that is known as mitochondrial potential. Measuring mitochondrial potential, which drives mitochondrial ATP synthesis (Nicholls and Budd, 2000), can assess mitochondrial function and integrity. Although early studies with isolated mitochondria have elaborately described mitochondrial properties, today's challenge is to understand the widespread roles of mitochondria and their interplay with other intracellular organelles. Live cell imaging of mitochondria, more than a decade ago, revealed these organelles are highly dynamic. Since then many researchers have focused on understanding the dynamism of these organelles (Detmer and Chan, 2007), as this property might be coupled to how mitochondria perform their many functions inside the cells. Therefore, techniques for imaging mitochondria are continually being improved to help further understand the structure/function relationship of these dynamic organelles.

Mitochondria are distributed throughout the cell and they exist in different sizes and forms that range from small granular to highly filamentous shapes. Although lack of

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mitochondrial dynamism is deleterious to cells, it is not understood how changes in mitochondrial morphology bring about change in mitochondrial function (reviewed in Detmer and Chan, 2007). The dynamics of mitochondrial forms within cells is controlled by specific molecules involved in mitochondrial fission and fusion, as well as those controlling the cytoskeletal structure, most importantly microtubules (De Vos et al., 2005). The steady-state fission-fusion events of mitochondria together with the motility of individual mitochondria pose serious challenges for high-resolution live cell imaging of mitochondria; this challenge has only been recently met with the advent of confocal microscopes with high-speed acquisition. This unit describes fluorescence-based confocal imaging approaches to study mitochondrial dynamics and function in live mammalian cells.

STRATEGIC PLANNING

Live cell imaging of mitochondria can be performed by tagging resident mitochondrial proteins with fluorescent proteins or by adding specific mitochondrial targeting sequences to fluorescent proteins (Okamoto et al., 2001). Commercially available dyes can also be used, although with limitations (Chen, 1988). Depending on the researcher's interest, there are various approaches for imaging mitochondria. For example, high-resolution three-dimensional imaging can provide details about mitochondrial structures and their association with organelles like the endoplasmic reticulum. Time-lapse imaging techniques allow aspects of mitochondrial dynamics to be studied quantitatively and qualitatively. It can also be used to visualize exchange of molecules, like mitochondrial DNA, between mitochondria undergoing fission and fusion events (Detmer and Chan, 2007). The physical and electrical continuity between mitochondria undergoing fission and fusion can also be studied using live cell imaging approaches employing FRAP and microirradiation protocols. Finally, mitochondrial potential can be quantified on a single cell or single mitochondrion based on incorporation of specific fluorescent dyes.

As mitochondria are an integral part of the cell death signaling machinery, their morphology is highly sensitive to the status of cellular health. A great deal of caution, therefore, is required to avoid causing any perturbations to this organelle during imaging. The points discussed below should always be considered when preparing samples for imaging mitochondria in order to optimize imaging conditions and to avoid artifacts. The protocols described here are optimized for adherent cells with 75% confluency. However, they can be modified as per the requirements for suspended cells.

Sample Preparation

1. When working with mitochondrial markers tagged to fluorescent proteins (FP), it is recommended to first make a stable cell line expressing the molecule of interest. This is because transient expression itself is a stress to cells, which might affect mitochondrial morphology. For example, we have found that the DsRed molecule targeted to the mitochondrial matrix causes mitochondrial fragmentation after 48 hr of transient transfection. For primary cultures where markers cannot be stably expressed, transient expression systems can be used to express molecules at low levels for short period of time. One can also choose to use fluorescent dyes to stain mitochondria for short-term experiments.

In the case of overexpression systems, transient or stable, precautions have to be taken to ensure that expression of the fluorescent molecule of interest is not altering mitochondrial morphology. This can be done by comparing mitochondrial morphology in the stable line and its parental line. The steps include: (1) staining the parental line (that is not expressing the mitochondrial FP) with a fluorescent mitochondrial dye (Mitotracker green etc.); (2) counting cells according to mitochondrial morphology as fragmented/intermediate/tubular (Karbowski et al., 2006); (3) counting cells according to

mitochondrial morphology as fragmented/ intermediate/tubular in the stable line expressing mitochondrial fluorescent protein; (4) comparing the distribution of mitochondrial morphology of the parental line to that of the cell population expressing the fluorescent mitochondrial molecule.

2. The medium used for imaging should always be pre-incubated in a 37°C incubator with 5% CO_2 for 30 to 60 min.

3. The pre-incubated medium should be used to replace the medium in which cells were grown in the imaging chambers. This has to be done immediately after the plated cells have been brought out of the incubator. Next, the medium in the imaging chamber has to be carefully overlayed with mineral oil that would form a meniscus on the medium. The level of the medium has to be high enough so that the oil meniscus is not close to the bottom of the chamber where cells are plated, which might kill the cells. The actual purpose of the mineral oil is to prevent CO_2 from escaping the medium, which would change the pH of the medium. CO_2-independent medium can be used to avoid use of mineral oil. However, this is not recommended for experiments demanding more than 1 hr. In this time, evaporation of water from the medium would concentrate the components of the medium, which in turn might affect mitochondrial morphology and function.

4. The stage warmer should be preset 30 to 45 min before commencement of the experiment. A properly calibrated temperature probe is mandatory. The calibration of the temperature probe should be checked by dipping the probe in two water baths maintained at different temperatures. This ensures that the monitored temperature at the stage is correct. To monitor the stage temperature, the temperature probe should be put at the top of the objective (but not touching it), exactly at the point where the specimen in the stage holder to be imaged will be held during imaging.

Choice of Mitochondrial Marker

As mitochondria are double membrane-bounded organelles, one needs to have knowledge about the localization of the molecule/dye of interest in the mitochondria for proper interpretation of imaging results. At the resolution of light microscopy, one cannot distinguish between the outer and inner membranes but can distinguish between the (outer/inner) membranes and the matrix. Therefore, the final outcome of experiments might differ with different choices of molecules to image mitochondria.

Although high-resolution *z* stacks can be acquired by using fluorescent dyes, time-lapse imaging for assessment of mitochondrial dynamics could be erroneous when performed with fluorescent dyes. This is because dye addition has the possibility of affecting mitochondrial behavior. The best and reproducible result is obtained by using fluorescent mitochondrial markers like mitoRFP/mitoGFP/mitoYFP. Use fluorescent molecules with the excitation peak at longer wavelengths to avoid the possibility of mitochondrial damage from more energetic shorter wavelengths. Only the stable lines expressing mitochondrial markers can be imaged for very long time periods, even more than 24 hr. By contrast, experiments using fluorescent dyes need to be performed within 30 to 45 min, after which the mitochondria usually fragment in response to the foreign chemical.

For photobleaching experiments, the mitochondrial fluorescent tag should be chosen such that it is bright and photostable under low illumination but photobleaches fast and almost irreversibly. The irreversibility of photo bleaching is very important because if the bleached molecule reverts to its fluorescent state it will contribute to the recovery. This would lead to erroneous conclusion about the physical mitochondrial process under investigation. This can be checked by performing the experiment, with the exact same configuration as chosen, but in a fixed sample. If the reversion of the bleached molecule is minimal there should be no recovery in the fixed sample.

BASIC PROTOCOL 1

HIGH-RESOLUTION *z*-STACK AND TIME-LAPSE IMAGING OF MITOCHONDRIA

Analyzing a *z*-stack across the depth of the cell provides steady-state three-dimensional information about mitochondrial morphology while time-lapse imaging allows changes in mitochondrial morphology (dynamics) to be studied over time. Figure 4.25.1 depicts a representation of a projection of *z*-stacks of mitochondria in a single cell.

Materials

Stably transfected cells expressing fluorescent-tagged molecule of interest *or* mitochondrial fluorescent dyes of interest
Chosen dye (see the Support Protocol)
Immersion oil

Labtek or Matek chambers
CO_2 incubator at 37°C
Microscope stage heater and temperature probe (e.g., Oka-lab)
Confocal laser scanning microscope, like Zeiss LSM510 or similar, with appropriate laser and filter sets required for imaging
63× Plan-Neofluar oil objective with high NA (1.4)

1. Plate stably transfected cells in Labtek/Matek chambers at least 10 to 16 hr before imaging.
2. Stain the cells with the chosen dye, if required (see the Support Protocol).
3. Prepare the sample (see Sample Preparation in Strategic Planning).
4. Preheat the microscope stage to 37°C. Turn on the appropriate laser at least 15 min prior to the experiment to give it time to stabilize.
5. Put a drop of immersion oil on the objective and fix the chamber to be used to the stage holder properly.

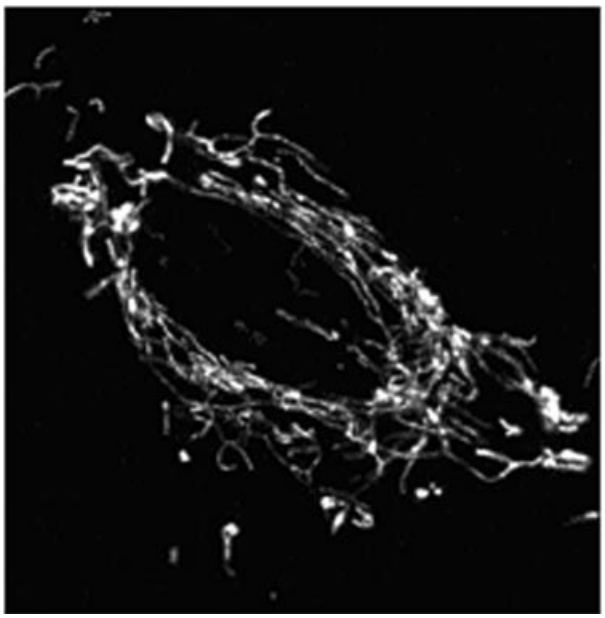

Figure 4.25.1 High-resolution image of mitochondria in a stable fibroblast cell line expressing mitoRFP. The image is a projection of *z*-stacks of mitochondria. A 543-nm HeNe laser was used for imaging and *z*-stacks were acquired following Basic Protocol 1. Imaging settings are listed in Table 4.25.1. The raw image has been converted into gray scale.

Table 4.25.1 Scanning Parameters for Acquiring High-Resolution *z*-Stacks of Mitochondria

Parameter	Value
Zoom[a]	2.5-3
Pixel size	1024 × 1024
Scan speed	7
Scan number for averaging	2
Pinhole	1 airy unit
Laser intensity	1%-20%
Detector gain	600

[a]High-resolution imaging of mitochondria would limit the investigator to scan one cell at a time, or part of a cell or even individual mitochondria. To image a whole cell the zoom factor (digital magnification) will have to be decided based on the size of the cell. A zoom factor of more than 5 is not recommended as this would cause the image to be pixilated, but this value can be used, if absolutely necessary, by adjusting other parameters.

6. Choose the appropriate configuration for the fluorophore to be imaged.

   To be able to do so one needs prior knowledge of the excitation and emission properties of the fluorophore (UNIT 21.5)

7. Choose a cell to be imaged by using the FAST scan button in minimum zoom.

   One can also quickly select a cell by looking through the eyepiece of the microscope with the proper filter. Selecting a cell, if not accomplished within few seconds, might cause bleaching of the fluorophore or photodamage to the mitochondria.

8. Adjust the laser intensity and detector gain according to the brightness of the sample in such a way that the signal from any pixel is not saturated (as checked in the palette in the LSM 510 systems).

   For quantitative imaging, this is essential, but for qualitative purposes some pixels may be allowed to cross the threshold to be able to detect other very faint pixels. This is because the fluorescent signal might not be distributed to all the mitochondria homogeneously, especially in certain conditions where normal mitochondrial morphology is perturbed.

9. Set the scanning parameters including zoom, pixel size, scan speed, scan number for averaging, pinhole, laser intensity, detector gain, etc. and scan a single section.

   Table 4.25.1 provides values for these parameters for proper imaging of mitochondria of a whole cell. If necessary, the parameters can be changed to yield better results.

10. If satisfied with the image, check for minimal bleaching.

    This is very important for time-lapse imaging. With laser power and scanning parameters being fixed, bleaching would depend on:

    (1) Scanning frequency: As mitochondrial movement per second is below the resolution limit of light microscope (as measured in neurons; De Vos et al., 2003), an imaging speed of 1 frame per second serves the purpose. One can further reduce the speed to 0.2 frames per second or 1 frame every 5 sec.

    (2) Number of scans: The total number of scans in a time series would depend on the duration of the experiment. For minimal bleaching, it is recommended to give more time between scans when intended to perform a longer time series experiment.

    The scanning parameters can be altered to reduce bleaching by enhancing scan speed and reducing averaging. Doing so will compromise the resolution. Therefore, a degree of trade

off between resolution and dynamics of mitochondrial structure has to be considered; the choice will be dependent on the goal of the experiment being performed.

Bleaching could arise during acquisition of z-stacks. This can be assessed by performing a time series where the time interval will be the same as the scan time of a single z-section. If there is no reduction in signal in this time, the parameters are suitable for z-sectioning. If not, the laser intensity and detector gain have to be adjusted according to the brightness of the sample.

11. Acquire the time series or *z*-stacks and save images.

    For acquiring overlapping z-stacks, the size of the optical slice should be twice than that of the z-interval, while for non-overlapping z-stacks, the optical slice should be close to that of the z-interval. For example, if pinhole size allows light from a 1-μm optical slice, the z-interval should be 0.5 μm for overlapping z-stacks and 1 μm for non-overlapping z-stacks. Oversampling might scan the same portion of the image more than once while undersampling might miss scanning portions. This could introduce errors in quantitative measurements like mitochondrial size, especially in case of tubular mitochondria.

    For long-term time series experiments, one should be aware of the drift in focus (due to thermal or other fluctuations) in the imaging setup and include autofocusing modules, like Multitime (Zeiss) for correcting the drift.

ALTERNATE PROTOCOL

IMAGING MITOCHONDRIAL MORPHOLOGY ALTERATIONS

The morphology of the mitochondria is influenced directly and indirectly by many factors. For example, nearly all the agents causing mitochondria-dependent cell death have been found to cause mitochondrial fragmentation (Karbowski and Youle, 2003). Recent studies are also being focused on identifying drugs that would directly impact the mitochondrial fission-fusion machinery and alter mitochondrial morphology (Cassidy-Stone et al., 2008). To image the effect of drugs or other chemicals on mitochondrial morphology/function, include the following steps for modifying acquisition of time series as mentioned in Basic Protocol 1. An example is illustrated in Figure 4.25.2, where time-lapse images have been acquired immediately after addition of microtubule depolarizing drug, nocodazole (5 μg/ml) to investigate the change in mitochondrial morphology after disrupting microtubules. Note the alteration in mitochondrial morphology from time 0 (before addition) to 30 min after addition.

Additional Materials *(also see Basic Protocol 1)*

Drug of choice (e.g., 5 μg/ml nocodazole)

1. Prepare the sample as in Basic Protocol 1 (or as in the Sample Preparation section of Strategic Planning), but do not overlay the sample medium with mineral oil.
2. Acquire 2 to 5 images of the chosen field.
3. Stop scanning and carefully remove the lid of the chamber.
4. Carefully aspirate 50% of the medium from the chamber using a pipet.
5. Add a volume equal to the volume removed of a 2× concentration of the drug to the chamber.

   The drug is added at twice the working concentration for the drug.

6. Carefully overlay with mineral oil.
7. Put the lid of the chamber back and quickly resume imaging.

   Care should be taken not to alter the plane of focus during the procedure of addition of drug. If the focus appears to be shifted, one has to quickly reset the focus keeping the last scanned image of the field as standard. The time period between stopping and resuming scanning has to be taken into account during the final analysis of the time series.

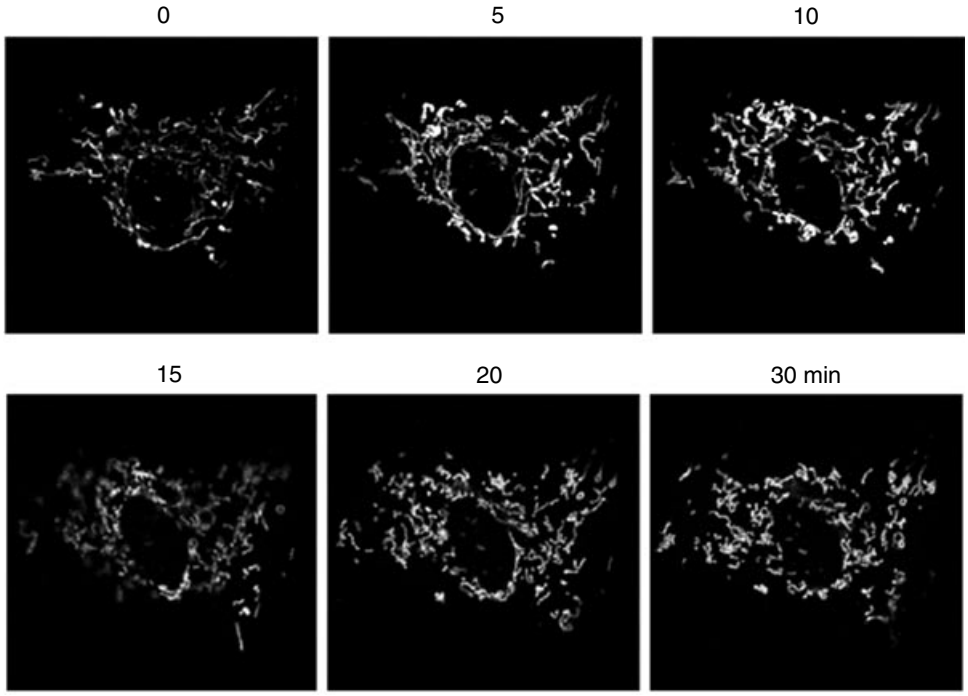

Figure 4.25.2 Time series of mitochondria after addition of nocodazole to depolymerize microtubules. Experiment was performed in a fibroblast line stably expressing mitoRFP. Time-lapse images were acquired following Basic Protocol 1 and the Alternate Protocol. The numbers represent time (in min) after addition of nocodazole. A 543-nm HeNe laser was used. The raw image has been converted into gray scale. Images were acquired using a 63× objective in zoom 2. Pinhole size was 2.5 airy units.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING ON MITOCHONDRIA

BASIC PROTOCOL 2

Fluorescence recovery after photobleaching (FRAP) is a powerful biophysical method that is used to understand the dynamics of molecules or structures in live cells. This method involves photobleaching of fluorescent molecules within a certain area of a cell and then monitoring recovery of fluorescence in the bleached zone by movement of molecules/organellar structures from the surrounding unbleached zone. For more detailed discussion of the theory of FRAP, please see *UNIT 21.1*. This experiment involves time-lapse imaging as it depends on the kinetics of mitochondria per se or that of a mitochondrial protein. Three types of information related to mitochondrial dynamics can be obtained from FRAP experiments depending on the time scale of recovery: (1) mobility of the fluorescent molecule—the time scale of recovery is in milliseconds and the recovery kinetics is a measure of diffusion of the fluorescent molecule of interest; (2) mitochondrial continuity: the time scale of recovery is from few seconds to 2 to 3 min depending on the area of bleach; this gives a measure of continuity of the mitochondrial compartment where the fluorescent molecule resides (please refer to Mitra et. al., 2009 for a specific example); (3) mitochondrial movement/dynamics (fission-fusion): the time scale is between 2 to 3 min to ∼1 hr; this recovery is due to the combination of mitochondrial motility and fission-fusion dynamics. The time scale of the FRAP experiment has to be chosen depending on the investigator's goal. The protocol here uses a microscope with faster scanning speed for measuring molecular mobility. Since the protocols are the same for all three options, they are mentioned under the same heading.

Materials

Confocal laser scanning microscope, like Zeiss LSM510 or similar, with appropriate laser and filter sets
63× Plan-Neofluar oil objective with high NA (1.4) is best for imaging mitochondria in tissue culture cells
Microscope stage heater and temperature probe

1. Repeat steps 1 to 7 from Basic Protocol 1.
2. Set the scanning parameters including mode, zoom, pixel size, scan speed, scan number for averaging, pinhole, laser intensity, detector gain, etc. Verify the set of parameters by doing a single scan each time after adjusting a parameter. Table 4.25.2 provides FRAP parameters for all the three options.

   Settings may be altered to improve imaging depending on the system.

3. *Define region of interest (ROI):* Bleaching will be performed and recovery will be followed in the specified ROI.

   Two important criteria about selection of an ROI should be considered.

   Size: One should have an expectation regarding the source of recovery of fluorescence (for all options) in the ROI before setting this parameter. The size of the ROI should not be more than 30% to 50% of the total signal area, so that the recovery of fluorescence in a given time from the unbleached zone (70% to 50%) can be appreciably detected. The time to reach maximal recovery by movement of molecule inside mitochondria (molecular mobility, mitochondrial continuity) or that of mitochondria themselves (mitochondrial mobility/dynamics) would depend on the available unbleached fluorescence that would contribute to the recovery. The ROI size would be dependent on which option of the FRAP one chooses to do. For molecular mobility, the ROI should be only around 0.5 × 0.5 μm; just enough to cover a small zone on an individual mitochondria (Table 4.25.2,

Table 4.25.2 Scanning Parameters for Performing FRAP Analysis on Mitochondria

Parameter	Molecular mobility	Mitochondrial continuity	Mitochondrial mobility/dynamics
Objective	63×	63×	63×
Zoom	1	2	2-3
Pixel size	512 × 80	512 × 512	512 × 512
Scan speed	9	8	7-8
Scan number for averaging	1	1	1
Pinhole[a]	Open	Open	Open
Laser intensity	0.5%-10%	0.5%-10%	0.5%-10%
Detector gain	600	600	600
Size of ROI	0.5 × 0.5 μm	Variable[b]	Variable[b]
Time interval	5 msec	1 sec	5 min
Bleaching time	7 μsec	>1 msec <1 sec	>1 msec <1 sec
Bleach iterations	2	5	5
Time-lapse imaging duration	500 msec	1-5 min	30 min to 1 hr

[a]The pinhole should be kept open to be able to collect light from the whole depth of the cell. If the pinhole were narrow, then movement of mitochondria in and out of the optical plane would introduce fluctuations in the reading. However, an open pinhole would compromise the resolution to a certain extent. Reduction of laser intensity can sometimes help to avoid out-of-focus signal, which, however, would depend on organization of mitochondria in the cell of interest.
[b]Refer to the text on ROIs.

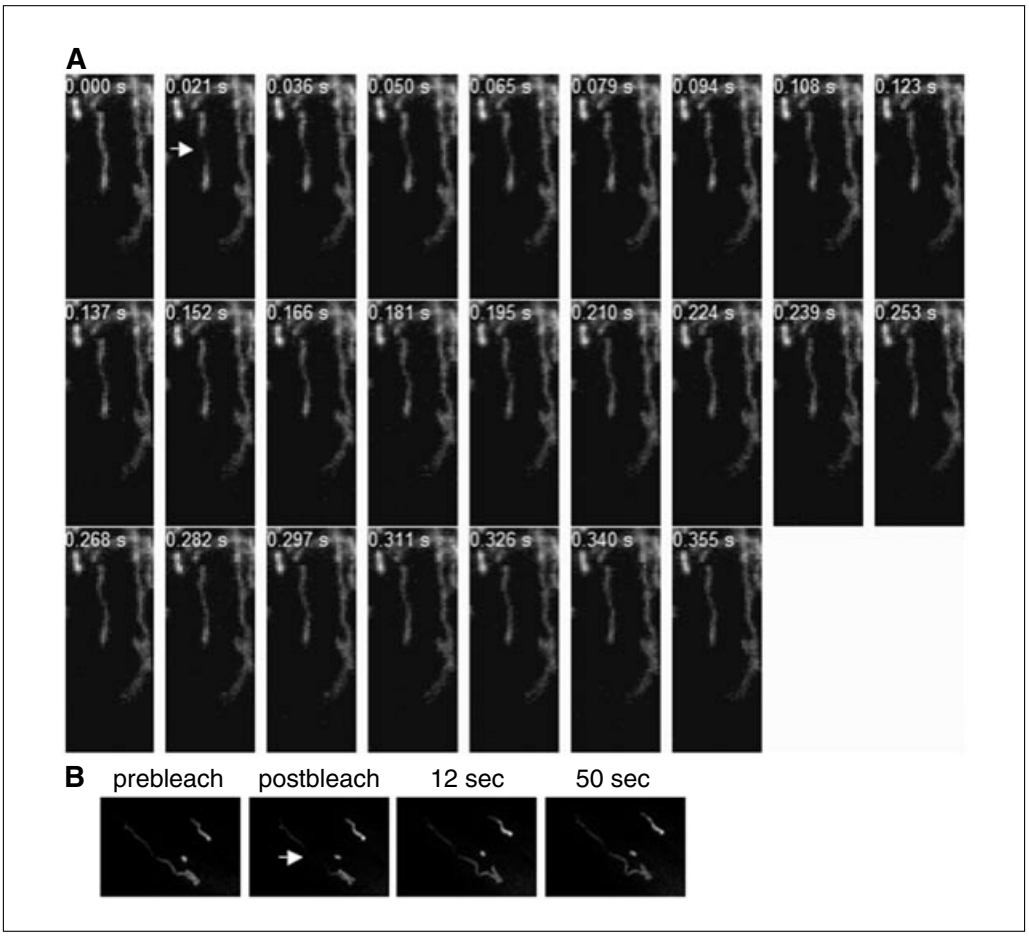

Figure 4.25.3 Fluorescence recovery after photobleaching on single mitochondria. Experiment was performed in a fibroblast line stably expressing mitoRFP following Basic Protocol 2. The time scale of recovery is in milliseconds. The arrow points to the bleached zone. Another mitochondrion in the field of view gives an assessment of overall bleaching during the recovery period. High-speed laser scanning confocal microscope (Zeiss LSM5 Duo) was used in **A** while a similar but slower microscope (Zeiss 510) was used in **B**. Prebleach and postbleach images depict mitochondria before and after the single bleaching pulse, respectively. A 543-nm HeNe laser line was used for imaging and other imaging settings used are mentioned in Table 4.25.2.

Fig. 4.25.4). For mitochondrial continuity and mobility/dynamics, one can decide to have the ROI spanning 5% to 2% of the cell either across the cell or locally (Fig. 4.25.3).

Placement: For continuity and mobility/dynamics measurements, the ROI should be placed in a region where the mitochondrial population is roughly uniformly distributed. In all the options, the ROI should be placed close to the center of the area of the total distributed signal. This means close to the center of the cell for continuity and dynamic measurements (Fig. 4.25.4) and the center of the mitochondrion of interest for molecular mobility studies (Fig. 4.25.3). This allows for movement of the fluorescence from all directions of the surrounding unbleached zone into the bleached zone. Wrong placement of the ROI, for example ROI placed to one side, might introduce errors in analyses and interpretation of the results.

4. *Standardize bleaching conditions:* Photobleaching is achieved by using maximum power of the of the same laser that is used for scanning. If the fluorescent molecule of choice is RFP, then it is recommended to use 458-, 488-, and 514-nm Argon lasers in addition to the less powerful 543-nm HeNe laser. As the RFP excitation spectrum with the peak at 543 nm also includes these wavelengths, the other lasers will enhance bleaching. For powerful 543-nm lasers, this might not be necessary.

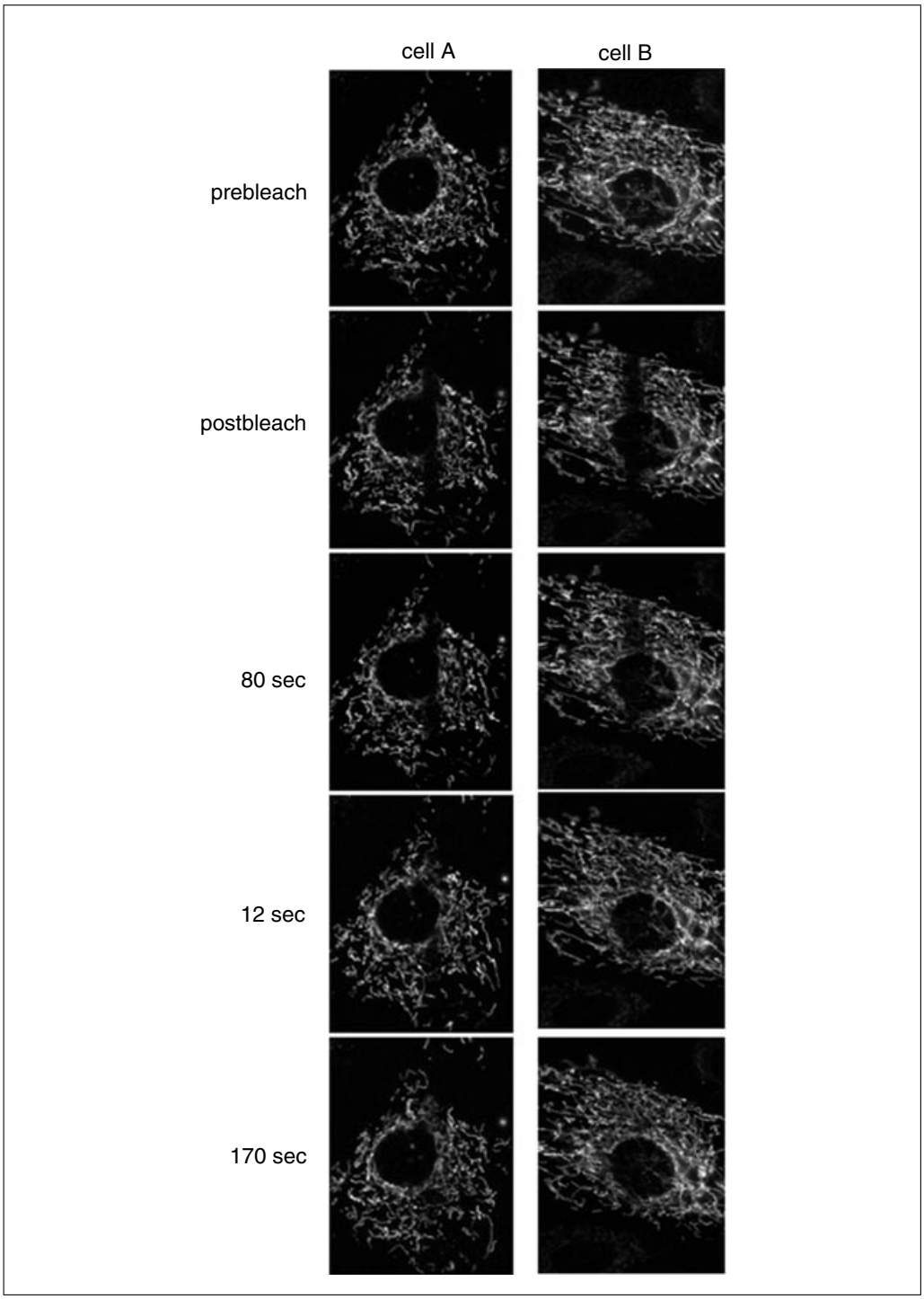

Figure 4.25.4 Fluorescence recovery after photobleaching on a mitochondrial population. Experiment was performed in a fibroblast line stably expressing mitoRFP following Basic Protocol 2. Prebleach and postbleach images depict mitochondria before and after the single bleaching pulse, respectively. The numbers represent time after post bleach in seconds. Two cells, A and B, have been shown to compare different recovery kinetics. A 543-nm HeNe laser line was used for imaging and other imaging settings used is mentioned in Table 4.25.2.

In continuity and mobility/dynamics measurements, bleaching should be as fast and as complete as possible; this can be increased by increasing the number of bleaching iterations; for molecular mobility measurements where the recovery kinetics is in milliseconds, bleaching should be faster and restricted to 20% to 40% of the initial fluorescence intensity. However, one should consider a trade off between the number of bleaching iterations and bleaching time. Bleaching for longer than necessary could damage mitochondria.

5. *Set the time interval and total imaging time:* These parameters depend on the option chosen.

   Scanning in short intervals would necessitate faster scanning. This may be achieved by restricting the scanning area to the zone of interest (Fig. 4.25.3A).

6. Acquire and save the time series to be analyzed when required.

   The quantitative analysis can be performed according to Goodwin and Kenworthy (2005). The number of recovery curves required for statistically significant analyses should be between 10 to 20. The data may be used to calculate half-time of recovery (t1/2), mobile and immobile fractions of the fluorescent molecule (see UNIT 21.1), diffusion coefficients (Partikian et al., 1998), and continuity parameters (Karbowski et al., 2006). An example of analysis of FRAP data has been provided in Figures 4.25.4 and 4.25.5. The figures depict analyses of mitochondrial connectivity in each of the two cells; bleaching was performed in the long ROI and the recovery in the small ROIs in each cell is compared to the recovery in the long ROI of the same cell. In cell A, individual ROIs appear to have different recovery kinetics while in cell B, individual ROIs have similar kinetics.

   The analysis will include correction for overall photobleaching that might have occurred during acquisition of the time series and total fluorescence that could contribute to the recovery. As mitochondria usually form small or large networks sharing their contents and continuously rearrange the branching points of the networks, it is very difficult to

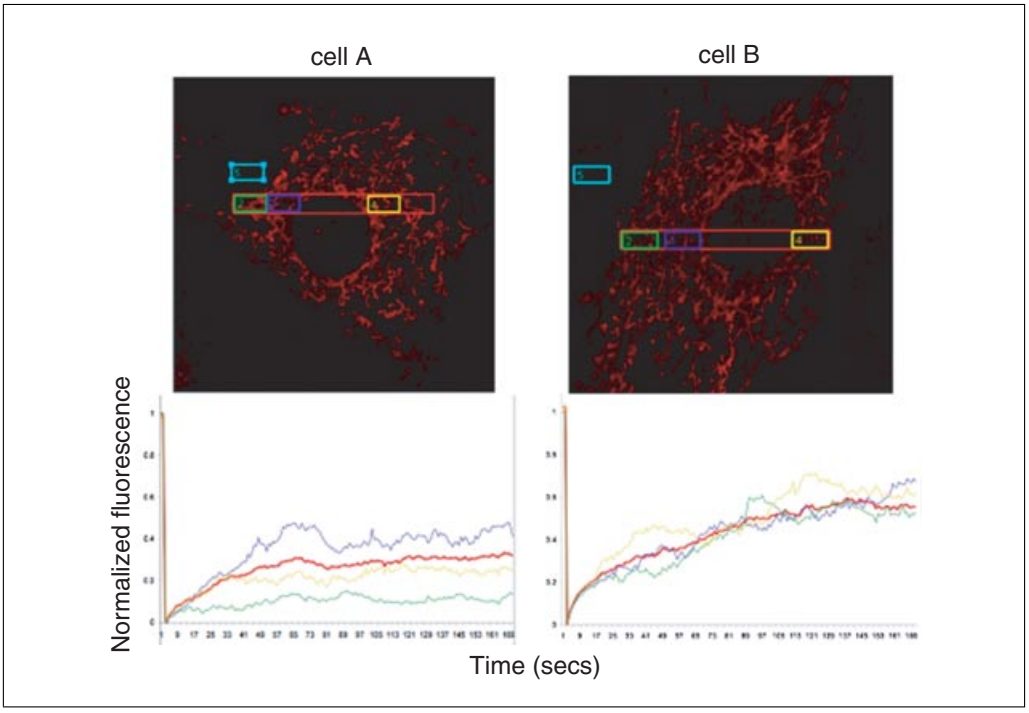

Figure 4.25.5 Analysis of connectivity by FRAP on a mitochondrial population. Images of cell A/B of Figure 4.25.4 were analyzed according to Goodwin and Kenworthy (2005). Different ROIs are color-coded corresponding to the associated graph showing recovery kinetics. The signal in the blue ROI was used to subtract the background signal. Signal was bleached in the red ROIs and recovery was monitored in all the ROIs. The analyses here do not include correction for bleaching and are normalized by the initial fluorescence. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

have a knowledge of the total source of unbleached fluorescence that could contribute to the recovery. Depending on the analysis performed, the total source of unbleached fluorescence factor might be considered constant or ignored. For example in molecular mobility, if the individual mitochondrion appears to form a part of the network, then it is recommended not to consider the factor. However, it is recommended that this kind of an analysis be performed on mitochondria near the edge of the cell where there will very few separated mitochondrial elements (Fig. 4.25.3B). In the example of connectivity analysis provided in Figure 4.25.5, the normalization by the total fluorescence has been ignored. In this case, the alternative is to consider the total mitochondrial fluorescence as the source of recovery (which may not be the case in reality).

FRAP on mitochondria to analyze mitochondrial connectivity or diffusion of mitochondrial molecule is complicated by the motility of the organelles. This problem cannot be avoided, but some verification has to be done before proceeding for image analyses. Careful examination of the time series images is required for any of the three possible errors that could have been introduced during image acquisition: (1) sudden appearance of one or more motile mitochondria in the ROI considered; (2) mitochondria shifting or moving away, thus making the signal in the ROI unrepresentative of the experimental result for molecular mobility measurement; (3) the whole cell of interest moving during acquiring the time series for continuity and mobility/dynamics measurements. Any time series having one or more of these errors have to be discarded from the analyses.

BASIC PROTOCOL 3

MICROIRRADIATION ASSAY TO ASSESS ELECTRICAL CONTINUITY IN MITOCHONDRIA

Knowledge of mitochondrial biochemistry and energetic properties has been used to design fluorescent dyes (commercially available from Molecular Probes) to stain mitochondria in live cells (*UNIT 4.4* and Support Protocol 1 covers staining of mitochondria with these dyes). The fluorescent derivatives of certain cationic lipophilic compounds (i.e., TMRE) concentrate inside the mitochondria that maintain inner membrane potential. Therefore, uptake of TMRE can be used to quantitatively determine mitochondrial transmembrane potential. TMRE-loaded mitochondria are highly sensitive to laser scanning, which is reflected in the spontaneous depolarization that can happen when imaging TMRE loaded mitochondria in cells (Bunting, 1992). This phenomenon can be manipulated in a controlled fashion using a high-intensity laser to irradiate and thus depolarize TMRE-loaded mitochondria, resulting in immediate loss of TMRE from the irradiated mitochondria (Amchenkova et al., 1988). Directed irradiation in an ROI can cause controlled depolarization very locally in an individual mitochondrion in a cell. This local depolarization will rapidly spread from the irradiated point to mitochondrial elements in electrical continuity to the irradiated point (Fig. 4.25.6). Therefore, one can assess the extent of electrical continuity of mitochondria in an individual by micro-irradiating one point in a mitochondrion and monitoring, by time-lapse imaging, the spread of depolarization to other electrically continuous mitochondria.

Materials

Microscope stage heater and temperature probe
Laser scanning confocal microscope with (1) a 543-nm laser line and appropriate filter sets for imaging rhodamine and (2) a 2-photon chameleon laser
63× plan neofluar objective
Power meter (from Coherent)

Additional reagents and equipment for staining with TMRE (Support Protocol)

1. Preheat the microscope stage to 37°C and turn on the 543-nm HeNe laser and the 2-photon chameleon laser.
2. Stain with TMRE as in the Support Protocol.

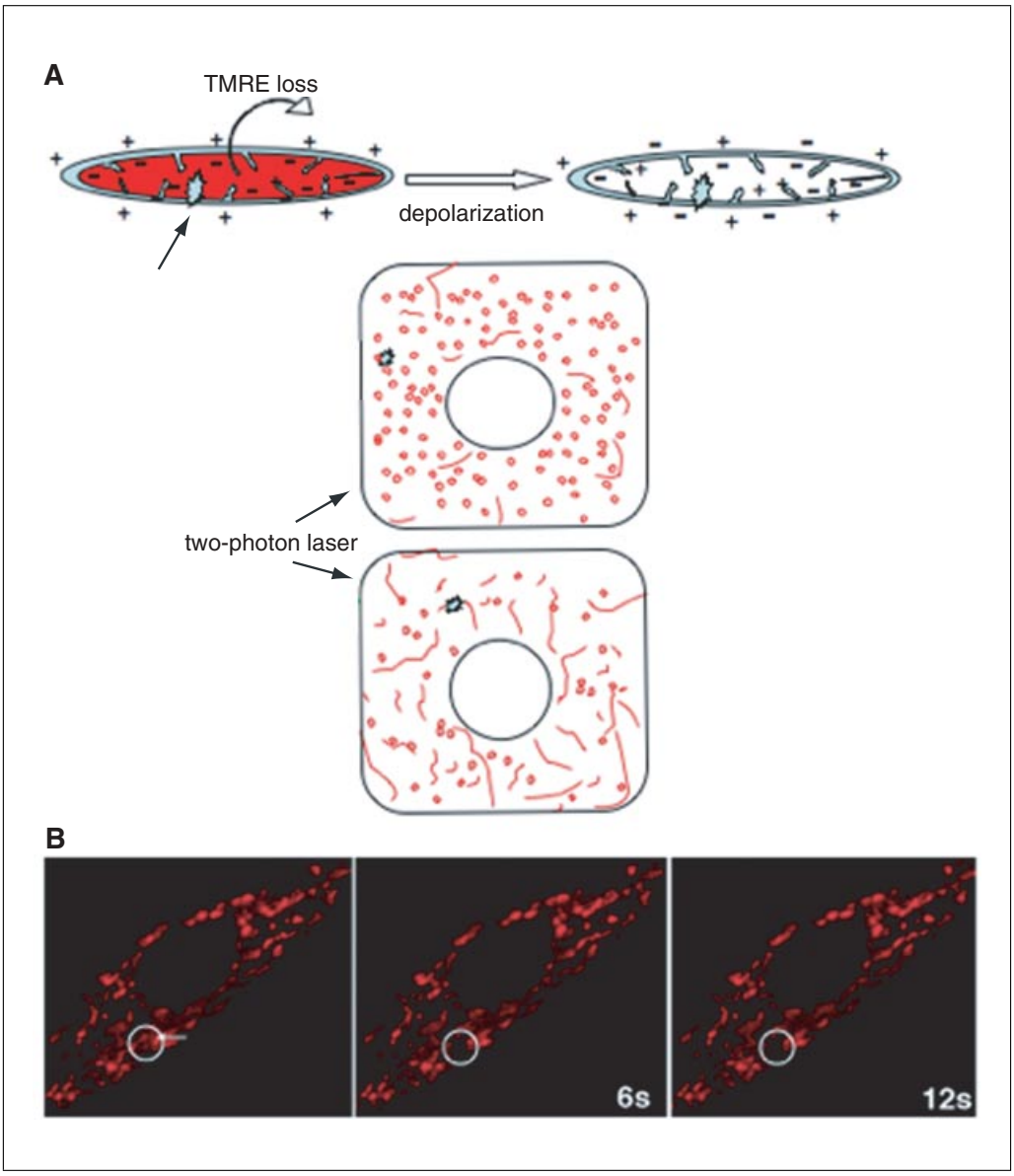

Figure 4.25.6 Microirradiation of TMRE-loaded mitochondria. (**A**) Depicts schematic representation of the microirradiation protocol. Mitochondria maintaining transmembrane potential (of charge) incorporate TMRE in the matrix. After being irradiated (green arrows) by the 2-photon laser locally, loss of TMRE occurs from the whole mitochondria due to depolarization triggered by the laser (as depicted for a single mitochondria in the upper panel and inside a cell in the lower panels). When this experiment is performed in cells, only single mitochondria are targeted for irradiation. (**B**) Shows images of a microirradiation experiment in a fibroblast where the arrow points to the site of irradiation. Images were acquired following Basic Protocol 3. Loss of TMRE signal is seen in the circled area in the time mentioned in seconds. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

3. Repeat steps 1 to 7 from Basic Protocol 1.
4. *Set up imaging settings:* Use the 543-nm laser line for imaging. To image TMRE, use settings for Rhodamine. An open pinhole is used to collect signal from the whole cell. Choose zoom factor 4 for high-resolution imaging. Scanning speed and averaging can be decided by the investigator to obtain best possible resolution.
5. *Define ROI:* To be able to cause microirradiation for assessing electrical continuity one should choose an ROI covering only a small area of the mitochondrial population. The ROI should not exceed an area of 1 × 1–μm.

6. *Set up microirradiation settings*: Chose the 2-photon chameleon laser as the bleaching laser for causing microirradiation. Tune the laser to 800 nm. Use 10% to 20% of the laser power and appropriate filters to cut off longer wavelengths. Set number of iterations to 5. Set optimum imaging speed to one frame per second to be able to monitor spread of depolarization.

   All parameters, including the objective, should be kept constant for reproducible microirradiation, as each will affect the net power delivered to unit area of the sample. The choice of the microirradiating laser depends on the power of the laser. As the physics behind 2-photon excitement requires the laser to be highly powerful (Piston, 1999), this type of laser has been used in this protocol. Proper alignment of the 2-photon laser is critical to achieve the required power for microirradiation (see Dickinson, 2005 for details about 2-photon microscopy). The power at the rear end of the objective should be optimally ~60 mW, as measured with the power meter. Any other laser achieving the power requirement can also be used to microirradiate.

7. *Assess damage to the cell and mitochondria:* The optimal microirradiation settings should aim at causing mitochondrial depolarization and loss of TMRE within the ROI but not damage the cell or fragment the irradiated mitochondrion.

   The damage to the cell can be assessed by performing time-lapse imaging with the transmitted light/DIC, with the microirradiation setup. This time series has to be examined carefully to confirm that no unwanted formation has occurred at the point of irradiation and the cell has not swelled in the time scale of the experiment, both of which would indicate cell damage.

   Mitochondrial damage can be assessed by performing the microirradiation time-lapse experiment on cells expressing a mitochondrial fluorescent protein marker. Observing the mitochondria at the irradiated zone carefully, in the microirradiation time series, one should confirm that the microirradiation does not fragment the mitochondrion in the time scale of the experiment, which could be an indication of damage.

   If there is any indication of cellular or mitochondrial damage, the power of microirradiation has to be minimized by reducing the bleaching iterations or the power of the laser.

8. Acquire the microirradiation time series and save images to be analyzed when required.

SUPPORT PROTOCOL

STAINING MITOCHONDRIA IN LIVE CELLS TO ASSESS MITOCHONDRIAL FUNCTION BY IMAGING

Knowledge of the mitochondrial lipid environment has been used to synthesize fluorescent compounds that stain mitochondria in live cells. This protocol describes staining and imaging of live tissue culture cells using the mitochondrial labels TMRE, JC-1, mitotracker green, or Picogreen. Mitotracker Green and Non Acridyl Orange are such compounds that can be used to measure mitochondrial mass. Therefore, from the same cells stained with both Mitotracker green and TMRE (for mitochondrial potential, see Basic Protocol 3), one can assess mitochondrial potential per unit of mitochondrial mass on a single-cell basis or in single mitochondria. Compounds like JC-1 are also taken up by the mitochondria by the virtue of their transmembrane potential. This dye, which gets enriched in the high potential zones of mitochondria, can form aggregates that have altered emission spectra (Smiley et al., 1991). Moreover, mitochondrial DNA can be visualized with the DNA binding dye PicoGreen (Ashley et al., 2005; Fig. 4.25.7).

Additional Materials *(also see Basic Protocol 1)*

Appropriate growth medium
Respective dye: e.g., TMRE/Mitotracker Green/JC-1/Picogreen (Molecular Probes)
DMSO
Mineral oil

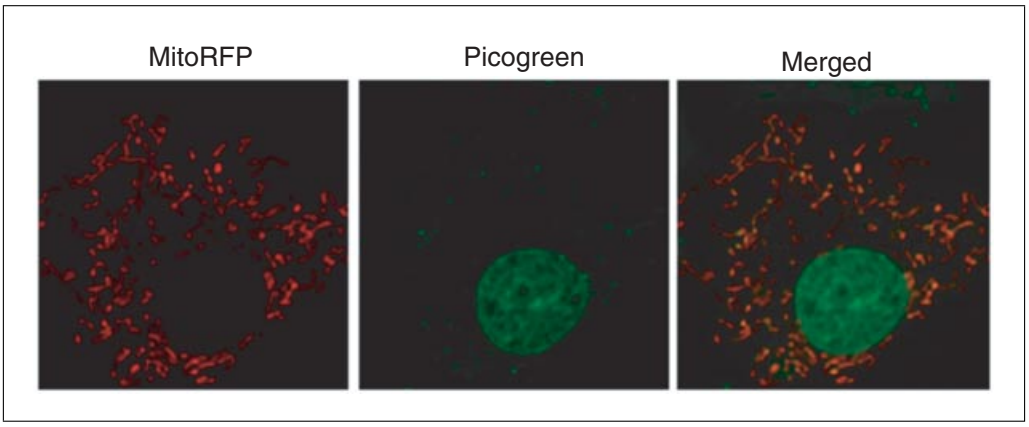

Figure 4.25.7 Picogreen staining of mitochondrial DNA. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

37°C CO_2 incubator
Fluorescence microscope with proper filters for Rhodamine and Fluorescein

1. Plate cells in Labtek or Matek chambers with 75% confluency and perform the experimental procedure as required on the cells at least 16 hr after plating.
2. Incubate required volume of growth medium in the CO_2 incubator 1 hr at 37°C.
3. Thaw an aliquot of the respective dye and make the staining solution by diluting in the pre-incubated medium. Follow Table 4.25.3 for working concentrations.

   Each dye solution should be made in DMSO and maintained as small aliquots at −20°C. Freezing and thawing more than 5 to 6 times is not recommended.
4. Add a generous volume of the staining solution to the cells and incubate for 15 min in 37°C incubator.
5. Wash the cells three times, each time with pre-incubated medium in the same volume as that of the added staining solution (from step 4).
6. For double staining of Mitotracker Green and TMRE (to assess mitochondrial potential per unit mass), add diluted TMRE solution to cells already stained with Mitotracker green at this point and incubate for 15 min more.

   The following criteria have to be confirmed:

   (1) TMRE loading is below the quenching limits: When mitochondria are overloaded with TMRE a phenomenon of self-quenching occurs between TMRE molecules (Ward et al., 2000). This reduces the net TMRE signal obtained from mitochondria and thus will produce erroneous results. The concentration of TMRE mentioned here is below quenching limits but one should check this for every experimental set up as mentioned below.

   (2) No FRET occurs between Mitotracker Green and TMRE: fluorescence resonance energy transfer is a process by which energy is transferred from a donor molecule to an acceptor molecule with an overlapping excitation spectrum with the donor emission spectrum (UNIT 17.1). It has been reported that at certain concentrations FRET occurs from Mitotracker Green to TMRE molecules inside the mitochondria (Elmore et al., 2004). This will reduce the green signal in the presence of TMRE. Although the protocol involves much lower concentrations of both the dyes than necessary to undergo FRET, it is recommended this be verified in every experimental set up.

   Both of these caveats can be easily verified by performing time-lapse imaging of a double-stained sample after adding FCCP as mentioned in the Alternate Protocol. Addition of

Table 4.25.3 Working Concentration for Various Mitochondrial Fluorescent Dyes

Fluorescent dye	Working concentrations
TMRE	25-50 nM
Mitotracker Green	50-100 mM
Mitotracker red 633i[a,b]	250 nM
JC-1[c]	5 nM
PicoGreen[d]	1:500 dilution

[a]Overstaining results in the incorporation even in the endoplasmic reticulum.
[b]This dye and some others not mentioned here, are dependent on the mitochondrial potential to variable extents. Please refer to the relevant literature for the correct choice of dyes.
[c]Although the dye JC-1 can be used for qualitative purposes to assess heterogeneity in mitochondrial potential, it does not yield quantitative results and also often causes distortion in mitochondrial morphology. The dye forms particulate structures in solution; therefore, it is recommended to do a serial dilution after spinning down the solution each time. Otherwise, the particulate structure will deposit down on the bottom of the chamber during staining and the cells close to the particulate matter will incorporate more of the dye, yielding very erroneous results.
[d]Dilution is from a stock bought from Molecular Probes (P7581). Mitochondrial DNA would appear as cytoplasmic dots if optimally stained with this stain as seen in Figure 4.25.5. It is always recommended to perform dual staining with another mitochondrial marker or dye as cytoplasmic dots could also be due to mycoplasma infections.

FCCP would cause mitochondrial depolarization and loss of TMRE from the mitochondria. Therefore, if depolarization by FCCP reduces the mitochondrial red signal, the TMRE used is below the quenching level. On the other hand, if depolarization by FCCP increases the intensity of TMRE signal from mitochondria, it is an indication that TMRE loading is beyond the quenching levels. In this case, the working TMRE concentration has to be lowered and the test has to be repeated further. If the loss of TMRE signal by FCCP does not enhance the green signal from Mitotracker Green, then it is an indication of lack of energy transfer between the two fluorescent dyes. If addition of FCCP increases the signal from Mitotracker Green (indicative of FRET), then the working concentrations of the dyes have to be reduced and the test has to be repeated further.

In immortalized cells, an alternative for normalizing the mitochondrial mass could be replacing Mitotracker Green with stably expressing mitoGFP. This fluorescent molecule has a similar spectrum to that of Mitotracker Green and resides in the mitochondrial matrix. For this purpose, one needs to transfect mitoGFP into the cell line and select a stable clone that will be expressing mitoGFP equally in all the cells derived from the clone.

It is recommended to always include a control for hyperpolarization and depolarization in this analysis. Oligomycin would cause hyperpolarization by reducing mitochondrial ATP synthase activity and the ionophore FCCP would cause depolarization (Nicholls and Ward, 2000). As the assessment of mitochondrial potential by this kind of an analysis is done on a comparative scale, the samples treated with oligomycin and FCCP would serve to define the uppermost and lowermost limits in the scale of detection.

7. Repeat step 5.

8. Add pre-incubated medium and layer with mineral oil on top of the medium.

   Add enough medium to ensure that the mineral oil meniscus does not touch the bottom of the chamber where cells are coated.

Table 4.25.4 Imaging Parameters for Measuring Mitochondrial Potential per Unit Mass

Parameter	Individual cell	Individual mitochondria
Objective	40×	63×
Zoom	0.7	2-3
Scan speed	8	8
Averaging	2	2
Optical slice	Open	1 μm
z-section	———	0.5 μm
Laser power[a]	1%-10%	1%-10%

[a]The TMRE imaging of mitochondria at zoom 2 or 3 should be done very cautiously, as high laser power could quickly photodamage TMRE-loaded mitochondria (Bunting, 1992). Therefore, for optimum results, scanning laser intensity of the both the lasers should be kept as low as possible.

9. Choose the appropriate parameters for imaging, as is listed in Table 4.25.4.

   For assessing mitochondrial potential per unit mass, the configuration of filters for imaging should be chosen so that they restrict bleed through from one channel to the other in cells stained with both Mitotracker Green and TMRE. Adjust the laser intensity and detector gain according to the brightness of the sample in such a way so that the signal from any pixel is not saturated. Follow Table 4.25.4 for setting imaging parameters depending on whether the aim is to detect total mitochondrial potential per cell or that of individual mitochondria in single cells. Parameters can be altered for better imaging depending on the system.

10. Perform imaging as soon as possible according to the relevant set of imaging parameters.

    Beyond a period of 30 to 45 min, the mitochondria might start fragmenting leading to erroneous results.

    There could be variations in the sensitivity level according to the cell type and one should make a note of that. Generally, more sensitive cells would allow less time before mitochondria fragment and alter potential as an indication of a death trigger. Therefore, everything should be ready before the experiment and one should be quick in finishing all the scans.

11. Save the images and analyze the images at your convenience.

    The ratio of TMRE/Mitotracker Green is an assessment of mitochondrial potential in arbitrary units.

COMMENTARY

Background Information

The name mitochondria is a fusion of the Greek words "mito" meaning filaments and "chondro" meaning grains. The length of each mitochondrion can extend from 500 nm to 1 mm or more, the average diameter being 500 nm (Detmer and Chan, 2007). Contributions of electron microscopists aided in the detailed understanding of mitochondrial structure while biochemical analyses of isolated mitochondria identified the organelle as a seat of intermediate metabolism. In this era of live cell imaging, mitochondrial biologists are motivated to understand the structure-function relationship of mitochondria in live cells.

Imaging of mitochondria in live yeast and mammalial cells has been made possible by designing vectors expressing fluorescent proteins that are tagged to either a resident mitochondrial protein or any particular mitochondrial targeting sequence, targeting the fluorescent protein into any of the three mitochondrial compartments. Detailed strategy to that end in

yeast can be found in Okamoto et al. (2001) and mammals in Rizzuto et al. (1996). The latest generation of fluorescent protein variants is discussed in *UNIT 21.5*. The targeted fluorescent protein essentially enables the researchers to visualize the behavior of mitochondria within cells and/or study the behavior of the particular fluorescently tagged mitochondrial protein.

High-resolution live cell imaging of mitochondria aided in understanding the correlation of mitochondrial fragmentation and physiological properties of apoptotic cytochrome *c* release (Frank et al., 2001) or ROS production (You et al., 2006). The varied mitochondrial morphology in a population of cells can be quantitated from static images as fragmented, intermediate, or tubular according to the predominant mitochondrial morphology in an individual cell. However, in some extreme instances when mitochondria could also appear as clumps, it is erroneous to include them in any of the above three categories of mitochondrial morphology. These clumps could arise from clumping of fragmented mitochondria or collapse of a fused form of mitochondria. Therefore, in these situations, it is critical to perform FRAP analysis of any mitochondrial matrix molecule. This assay provides important measurements for mitochondrial continuity that would arise in fused mitochondrial structures. The assay could also be modified in a variety of aspects to understand mitochondrial dynamism. The interpretation of the recovery after photobleaching has to be carefully performed where the time scale of recovery is crucial for highly mobile organelles like mitochondria; failing to appreciate this property might yield confusing results. Furthermore, the alteration of recovery kinetics in a FRAP study for mitochondrial connectivity or motility might be contributed to by altered diffusing properties of the matrix molecule of interest. This has to be verified by performing FRAPs in individual mitochondria and any statistically significant change has to be included in the analysis of matrix continuity. To capture the complete kinetics of the recovery of the matrix molecule special microscopes are required with super fast scanning modules, like LSM5 Duo or Nikon epifluorescence microscope with super-fast integration time of the camera and photomultipliers attached to the microscope (Partikian et al., 1998). This will allow very fast photobleaching on the order of microseconds (Fig. 4.25.4A) that is required for molecules with diffusion coefficient in the order of 2–3 $\times$ $10^{(-7)}$ cm^2/sec, as in mitochondrial matrix. Slower microscopes, with longer bleaching time, will spread the bleaching beyond the bleach box, therefore increasing the recovery time (Fig. 4.25.4B). This kind of analysis might be used in a comparative scale but the data should not be used to determine diffusion coefficient.

The microirradiation protocol of TMRE-loaded mitochondria was first designed by Amchenkova et al (1988), and a similar technique has been also used to study various other organelles (Berns, 2007). High laser power has been shown to introduce holes in mitochondria (Khodjakov et al., 2004), thus disrupting mitochondrial potential across the inner membrane. Laser-induced depolarization of TMRE-loaded mitochondria has also been shown to increase localized ROS production (O'Reilly et al., 2003). This experiment has to be performed with caution about the cellular damage during the experimentation (as discussed before) and controls have to be included each day of experimentation. When dealing with more than one cell type, proper controls have to be included for each cell type under examination. It has to be very well appreciated that in a controlled setup the irradiated cell, behaving normally within the small time span of the experiment, might eventually die. Therefore, long-term experimentation may not be performed on such a cell. The microirradiation experiment is important from the mitochondrial bioenergetics point of view, as this qualitatively addresses the electrical continuity of the mitochondria at any given time point that might have impact on the total ATP output from the mitochondrial system (Skulachev, 2001).

The staining of mitochondria with different commercially available dyes is routinely practiced and the background information may be obtained from *UNIT 4.4*.

Post-processing of image analysis is a critical step for proper interpretation of imaging data. Although this unit does not cover image analysis in detail, mention may be made of some software that will be helpful for the purpose. Among the proprietary software, Zeiss' own Image Examiner can be used to analyze laser scanning confocal images. A free downloadable version that can be used as a viewer is available on Zeiss' Web site (*http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/CAA2EF638EC5F0D3C1256ADF0050E2F1*). Volocity and Metamorph can also be used. An open source software called Image J (*http://rsbweb.nih.gov/ij/download.html*) is very helpful for all basic purposes. Relevant plugins can be included for advanced purposes.

Table 4.25.5 Troubleshooting Guide to Live Cell Mitochondrial Imaging

Problems	Possible causes[a]
z-stacks and time lapse	
Fragmented/clumped mitochondria	Cells are not healthy pH or temperature shock Mineral oil touching the cells High amount of lipid-based transfecting agents used Transient transfection of the mitochondrial marker Effects of over-expression of the protein Immensely high scanning laser power Use of high-energy/low-wavelength laser (413/405-nm etc) or any other UV laser
Immotile mitochondria	Cells are not healthy for any of the reasons mentioned above High speed of imaging
Projection of *z*-stacks is blurry	Oversampling along *z*-axis Wide pinhole High scanning speed Saturated signals Low power objective
FRAP	
No recovery	Dead cells Fragmented mitochondria Immotile mitochondria Bleach box covers more than 30% of the total signal Inappropriately low pinhole size Bleaching caused by high laser power
Bleached zone is more than the bleach box	Misalignment of the laser head and bleach box Slow bleaching
Not enough bleaching	Low laser power chosen for bleaching For RFP, 488-, 458-, and 514-nm lasers not chosen
Poor recovery curve	Very long time intervals set for monitoring recovery phase Signal saturated in the beginning Scanning laser causing bleaching Not enough bleaching by the bleaching laser(s) Bleached molecule reverting to the fluorescent stage Movement of mitochondria interfering with recovery
Microirradiation	
No loss of TMRE after microirradiation	No irradiation due to application of lower laser power Misalignment of the 2-photon laser Low iterations chosen for irradiation Low zoom factor chosen
Mitochondria shifting away during imaging *or* mitochondria fragmenting	Cell is swelling due to more than optimum irradiation
Increase in TMRE signal after microirradiation	TMRE loading is beyond quenching levels

continued

Table 4.25.5 Troubleshooting Guide to Live Cell Mitochondrial Imaging

Problems	Possible causes[a]
Staining for potential	
No staining	Mitochondria are absolutely depolarized because cells are not healthy (as mentioned above)
Poor resolution	Overstaining Inappropriate pinhole size Lack of proper washing after staining
The oligomycin/FCCP-treated samples not producing expected results as controls	Overload of TMRE beyond the quenching levels FRET between Mitotracker Green and TMRE Bleed through from detector channel for Mitotracker Green to that of TMRE
Loss of TMRE while scanning	Power of the scanning laser power is more than optimum
Fragmented mitochondria	Stained sample left for more than 30 to 45 min

[a]For solutions please refer to the corresponding sections.

One could also include self-written macros in the list of Image J plugins.

Troubleshooting

This section discusses the problems that might arise while or before imaging mitochondria in particular. The general troubleshooting for imaging per se will not be discussed here. The possible causes to any problem are very crisply mentioned here and are discussed in more detail in the relevant protocol. See Table 4.25.5 for a troubleshooting guide.

Anticipated Results

The protocols described in this unit will help the researcher to understand mitochondrial function by imaging. The precautions mentioned here for imaging mitochondria are very critical to be able to achieve the goal of establishing structure/function relationships of mitochondria. By the correct use of imaging parameters and choice of fluorophore, the investigator can not only acquire static mitochondrial images but also perform time-lapse imaging on mitochondria. Live cell imaging of mitochondria including the FRAP and microirradiation protocols can be used by investigators to study the dynamics of mitochondria in quantitative terms and can also be used to understand dynamics of individual proteins in mitochondria. For example, study of the fluorescently tagged intermembrane space protein cytochrome *c* by live cell imaging has uncovered many important aspects of apoptotic release of this protein into the cytosol (Goldstein et al., 2000). Live cell imaging studies of mitochondria imply that imaging of fluorescent-tagged versions of mitochondrial proteins have tremendous potential of unraveling many unexplored aspects of mitochondrial physiology and its crosstalk with other cellular compartments.

Time Considerations

Generation of a stably expressing cell line takes 1 to 2 months depending on the cell type. Staining with fluorescent dyes and sample preparation takes from 30 min to 2 hr. Stained samples should be imaged within 45 min. Acquiring each *z*-stack will take <1 min; it takes a little longer for very slow scan speeds. The total time for a time-lapse experiment would depend on the kinetics of the phenomenon to be observed. To observe mitochondrial dynamism, a time lapse of 2 to 3 min should be sufficient. Each FRAP assay will take from less than half a minute to an hour, depending on the goal of the experiment. Extent of electrical continuity can be captured within 15 sec or less.

Literature Cited

Amchenkova, A.A., Bakeeva, L.E., Chentsov, Y.S., Skulachev, V.P., and Zorov, D.B. 1988. Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes. *J. Cell Biol.* 107:481-495.

Ashley, N., Harris, D., and Poulton, J. 2005. Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining. *Exp. Cell Res.* 303:432-446.

Berns, M.W. 2007. A history of laser scissors (microbeams). *Methods Cell Biol.* 82:1-58.

Bunting, J.R. 1992. A test of the singlet oxygen mechanism of cationic dye photosensitization of mitochondrial damage. *Photochem. Photobiol.* 55:81-87.

Cassidy-Stone, A., Chipuk, J.E., Ingerman, E., Song, C., Yoo, C., Kuwana, T., Kurth, M.J., Shaw, J.T., Hinshaw, J.E., Green, D.R., and Nunnan, J. 2008. Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev. Cell* 14:193-204.

Chen, L.B. 1988. Mitochondrial membrane potential in living cells. *Annu. Rev. Cell Biol.* 4:155-181.

Detmer, S.A. and Chan, D.C. 2007. Functions and dysfunctions of mitochondrial dynamics. *Nat. Rev. Mol. Cell Biol.* 8:870-879.

De Vos, K.J., Sable, J., Miller, K.E., and Sheetz, M.P. 2003. Expression of phosphatidylinositol (4,5) bisphosphate-specific pleckstrin homology domains alters direction but not the level of axonal transport of mitochondria. *Mol. Biol. Cell* 14:3636-3649.

De Vos, K.J., Allan, V.J., Grierson, A.J., and Sheetz, M.P. 2005. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. *Curr. Biol.* 15:678-683.

Dickinson, M.E 2005. Multiphoton and multispectral laser scanning microscopy. *In* Live Cell Imaging: A Laboratory Manual. (R.D. Goldman and D.L. Spector, eds) pp. 281-301. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Elmore, S.P., Nishimura, Y., Qian, T., Herman, B., and Lemasters, J.J. 2004. Discrimination of depolarized from polarized mitochondria by confocal fluorescence resonance energy transfer. *Arch. Biochem. Biophys.* 422:145-152.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. 2001. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev. Cell* 1:515-525.

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. 2000. The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2:156-162.

Goodwin, J.S. and Kenworthy, A.K. 2005. Photobleaching approaches to investigate diffusional mobility and trafficking of Ras in living cells. *Methods* 37:154-164.

Karbowski, M. and Youle, R.J. 2003. Dynamics of mitochondrial morphology in healthy cells and during apoptosis. *Cell Death Differ.* 10:870-880.

Karbowski, M., Norris, K.L., Cleland, M.M., Jeong, S.Y., and Youle, R.J. 2006. Role of Bax and Bak in mitochondrial morphogenesis. *Nature* 443:658-662.

Khodjakov, A., Rieder, C., Mannella, C.A., and Kinnally, K.W. 2004. Laser micro-irradiation of mitochondria: Is there an amplified mitochondrial death signal in neural cells? *Mitochondrion* 3:217-227.

Mitra, K., Wunder, C., Roysam, B., Lin, G., and Lippincott-Schwartz, J. 2009. A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc. Natl. Acad. Sci. U.S.A.* 106:11960-11965.

Nicholls, D.G. and Budd, S.L. 2000. Mitochondria and neuronal survival. *Physiol. Rev.* 80:315-360.

Nicholls, D.G. and Ward, M.W. 2000. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: Mortality and millivolts. *Trends Neurosci.* 23:166-174.

Okamoto, K., Perlman, P.S., and Butow, R.A. 2001. Targeting of green fluorescent protein to mitochondria. *Methods Cell Biol.* 65:277-283.

O'Reilly, C.M., Fogarty, K.E., Drummond, R.M., Tuft, R.A., and Walsh J.V. Jr. 2003. Quantitative analysis of spontaneous mitochondrial depolarizations. *Biophys. J.* 85:3350-3357.

Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A.S. 1998. Rapid diffusion of green fluorescent protein in the mitochondrial matrix. *J. Cell Biol.* 140:821-829.

Piston, D.W. 1999. Imaging living cells and tissues by two-photon excitation microscopy. *Trends Cell Biol.* 9:66-69.

Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y., and Pozzan, T. 1996. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr. Biol.* 6:183-188.

Scheffler, I.E. 2001. A century of mitochondrial research: Achievements and perspectives. *Mitochondrion* 1:3-31.

Skulachev, V.P. 2001. Mitochondrial filaments and clusters as intracellular power-transmitting cables. *Trends Biochem. Sci.* 26:23-29.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D. Jr., and Chen, L.B. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc. Natl. Acad. Sci. U.S.A.* 88:3671-3675.

Ward, M.W., Rego, A.C., Frenguelli, B.G., and Nicholls, D.G. 2000. Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurosci.* 20:7208-7219.

You, T., Robotham, J.L., and Yoon, Y. 2006. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc. Natl. Acad. Sci. U.S.A.* 103:2653-2658.