Video Article

A Faster, High Resolution, mtPA-GFP-based Mitochondrial Fusion Assay Acquiring Kinetic Data of Multiple Cells in Parallel Using Confocal Microscopy

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Abstract

Mitochondrial fusion plays an essential role in mitochondrial calcium homeostasis, bioenergetics, autophagy and quality control. Fusion is quantified in living cells by photo-conversion of matrix targeted photoactivatable GFP (mtPAGFP) in a subset of mitochondria. The rate at which the photoconverted molecules equilibrate across the entire mitochondrial population is used as a measure of fusion activity. Thus far measurements were performed using a single cell time lapse approach, quantifying the equilibration in one cell over an hour. Here, we scale up and automate a previously published live cell method based on using mtPAGFP and a low concentration of TMRE (15 nm). This method involves photoactivating a small portion of the mitochondrial network, collecting highly resolved stacks of confocal sections every 15 min for 1 hour, and quantifying the change in signal intensity. Depending on several factors such as ease of finding PAGFP expressing cells, and the signal of the photoactivated regions, it is possible to collect around 10 cells within the 15 min intervals. This provides a significant improvement in the time efficiency of this assay while maintaining the highly resolved subcellular quantification as well as the kinetic parameters necessary to capture the detail of mitochondrial behavior in its native cytoarchitectural environment.

Mitochondrial dynamics play a role in many cellular processes including respiration, calcium regulation, and apoptosis[1,2,3,13]. The structure of the mitochondrial network affects the function of mitochondria, and the way they interact with the rest of the cell. Undergoing constant division and fusion, mitochondrial networks attain various shapes ranging from highly fused networks, to being more fragmented. Interestingly, Alzheimer's disease, Parkinson's disease, Charcot Marie Tooth 2A, and dominant optic atrophy have been correlated with altered mitochondrial morphology, namely fragmented networks[4,10,13]. Often times, upon fragmentation, mitochondria become depolarized, and upon accumulation this leads to impaired cell function[18]. Mitochondrial fission has been shown to signal a cell to progress toward apoptosis. It can also provide a mechanism by which to separate depolarized and inactive mitochondria to keep the bulk of the network robust[14]. Fusion of mitochondria, on the other hand, leads to sharing of matrix proteins, solutes, mtDNA and the electrochemical gradient, and also seems to prevent progression to apoptosis[9]. How fission and fusion of mitochondria affects cell homeostasis and ultimately the functioning of the organism needs further understanding, and therefore the continuous development and optimization of how to gather information on these phenomena is necessary.

Existing mitochondrial fusion assays have revealed various insights into mitochondrial physiology, each having its own advantages. The hybrid PEG fusion assay[7], mixes two populations of differently labeled cells (mtRFP and mtYFP), and analyzes the amount of mixing and colocalization of fluorophores in fused, multinucleated, cells. Although this method has yielded valuable information, not all cell types can fuse, and the conditions under which fusion is stimulated involves the use of toxic drugs that likely affect the normal fusion process. More recently, a cell free technique has been devised, using isolated mitochondria to observe fusion events based on a luciferase assay[1,5]. Two human cell lines are targeted with either the amino or a carboxy terminal part of Renilla luciferase along with a leucine zipper to ensure dimerization upon mixing. Mitochondria are isolated from each cell line, and fused. The fusion reaction can occur without the cytosol under physiological conditions in the presence of energy, appropriate temperature and inner mitochondrial membrane potential. Interestingly, the cytosol was found to modulate the extent of fusion, demonstrating that cell signaling regulates the fusion process [4,5]. This assay will be very useful for high throughput screening to identify components of the fusion machinery and also pharmacological compounds that may affect mitochondrial dynamics. However, more detailed whole cell mitochondrial assays will be needed to complement this in vitro assay to observe these events within a cellular environment.

A technique for monitoring whole-cell mitochondrial dynamics has been in use for some time and is based on a mitochondrially-targeted photoactivatable GFP (mtPAGFP)[6,11]. Upon expression of the mtPAGFP, a small portion of the mitochondrial network is photoactivated (10-20%), and the spread of the signal to the rest of the mitochondrial network is recorded every 15 minutes for 1 hour using time lapse confocal imaging. Each fusion event leads to a dilution of signal intensity, enabling quantification of the fusion rate. Although fusion and fission are continuously occurring in cells, this technique only monitors fusion as fission does not lead to a dilution of the PAGFP signal[6]. Co-labeling with low levels of TMRE (7-15 nM in INS1 cells) allows quantification of the membrane potential of mitochondria. When mitochondria are hyperpolarized they uptake more TMRE, and when they depolarize they lose the TMRE dye. Mitochondria that depolarize no longer have a sufficient membrane potential and tend not to fuse as efficiently if at all. Therefore, active fusing mitochondria can be tracked with these low levels of TMRE[9,15]. Accumulation of depolarized mitochondria that lack a TMRE signal may be a sign of phototoxicity or cell death. Higher concentrations of TMRE render mitochondria very sensitive to laser light, and therefore great care must be taken to avoid overlabeling with TMRE. If the effect of

depolarization of mitochondria is the topic of interest, a technique using slightly higher levels of TMRE and more intense laser light can be used to depolarize mitochondria in a controlled fashion (Mitra and Lippincott-Schwartz, 2010). To ensure that toxicity due to TMRE is not an issue, we suggest exposing loaded cells (3-15 nM TMRE) to the imaging parameters that will be used in the assay (perhaps 7 stacks of 6 optical sections in a row), and assessing cell health after 2 hours. If the mitochondria appear too fragmented and cells are dying, other mitochondrial markers, such as dsRED or Mitotracker red could be used instead of TMRE.

The mtPAGFP method has revealed details about mitochondrial network behavior that could not be visualized using other methods. For example, we now know that mitochondrial fusion can be full or transient, where matrix content can mix without changing the overall network morphology. Additionally, we know that the probability of fusion is independent of contact duration and organelle dimension, is influenced by organelle motility, membrane potential and history of previous fusion activity[8,15,16,17].

In this manuscript, we describe a methodology for scaling up the previously published protocol using mtPAGFP and 15nM TMRE[8] in order to examine multiple cells at a time and improve the time efficiency of data collection without sacrificing the subcellular resolution. This has been made possible by the use of an automated microscope stage, and programmable image acquisition software. Zen software from Zeiss allows the user to mark and track several designated cells expressing mtPAGFP. Each of these cells can be photoactivated in a particular region of interest, and stacks of confocal slices can be monitored for mtPAGFP signal as well as TMRE at specified intervals. Other confocal systems could be used to perform this protocol provided there is an automated stage that is programmable, an incubator with CO_2, and a means by which to photoactivate the PAGFP; either a multiphoton laser, or a 405 nm diode laser.

Video Link

The video component of this article can be found at https://www.jove.com/video/3991/

Protocol

1. Image Plate Preparation

1. Culture INS1 cells in RPMI media containing 10% standard fetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 5 mM $NaHCO_3$, 2 mM HEPES, 2 mM pyruvic acid, and 11 mM glucose to 80% confluence.
2. Trypsinize the INS1 cell cultures with 0.05% trypsin and plate them onto poly-*D*-lysine coated coverslip-bottomed imaging plates (30-40% confluence).
3. Allow for plates to reach 60-80% confluence (~2 days) and add mitochondrial matrix targeted (COXVIII) adenoviral PA-GFP for 24 hr (MOI=5). Exchange media and allow cells to grow for another 2 days before imaging.
4. On the day of imaging, add 7-15 nM TMRE to the imaging plates, and equilibrate for at least 45 min.
5. During this time turn on the incubator (stage top incubator has been used here) and allow the microscope to equilibrate to 37 °C for about 1 hour. Turn on the 5% CO_2.

2. Imaging Parameters for the Zeiss LSM 710 Confocal Microscope

1. After the microscope has equilibrated, under the Acquisition tab, click on "show manual tools", open the imaging set up panel, and choose "channel mode" and switch track every "frame".
2. In the light path panel, choose LSM and channel mode. Working from the specimen icon upward, select "rear", MBS 690+, and MBS 488. At the bottom of the panel, choose "T-PMT" to enable visualization of the bright field or DIC channel.
3. Finish adjusting the light path parameters by setting the range for the GFP dye to 490-540 nm and to 580-700 nm for the TMRE dye.
4. In the acquisition mode, use a 512 × 512 pixel scan field, average 4x, and choose a zoom factor (which you may want to keep consistent for calibration purposes).

3. Optimizing the Imaging Parameters

1. Using the plan apochromat 100x (1.4 NA) objective lens, focus on your cells with the halogen light, not fluorescent light, to protect mitochondria from phototoxicity.
2. Screen for PA-GFP expressing cells using a maximally open pinhole and scanning to find the cells that are brighter green.
3. Find the lowest power of the 2-photon laser needed to activate the PA-GFP and optimize the imaging parameters ensuring that the signal does not saturate the detector. Check that the PA-GFP signal colocalizes with the TMRE signal for a few z-series. Loss of the TMRE signal indicates mitochondrial depolarization or phototoxicity, and cells exhibiting these characteristics should not be used for analysis.
4. Find a PA-GFP expressing cell and specify a zoom factor.
5. Set the z-series range to collect 6 slices (this range will need to satisfy all 10 cells unless a specific z-focus is set at each position).
6. Save the imaging method for each cell (cell 1, cell 2 etc.).

4. Adjust the Automated Portion of the Program and Designate the 10 Cells You Will Follow for the 1 Hour Mitochondrial Fusion Assay

1. On the left panel of the multitime window, select the "saving" panel, and specify the location where files will be saved.
2. In the "Acquisition" panel, load the acquisition method saved for cell 1 in the scan configuration and check the z stack box. Also choose "marked Z- middle of z stack".

3. In the "blocks" panel, select "single block at each location". Click on "add blocks" for each interval to be measured.
4. In the "timing" panel, select "wait interval" and type "0" in the "wait interval before block at first location only". Blocks 2-4 will have "15 min" in this section.
5. In the location panel, choose "move focus to load position between locations" and "load scan config when 'move to loc' or 'next loc' clicked. Under the "edit locations list" choose "clear all" and then select "multiple locations motorized stage".
6. Under the "Bleach" panel, click the "bleach" box, and designate the configuration file in the "config" pull down menu, as designated in the window of the main software. Then, in the "bleaching" window, save the appropriate photoactivation method. In the regions panel, choose the ROI for this particular cell, and add this ROI to the multitime by choosing "add current region to ROI list" window. Be sure to select the same location in the "ROI" pull down menu.

For the timing to work properly and for each cell to have a 15 minute interval, two methods need to be saved. In the list of blocks, the first one will have the "real" photoactivation configuration, and the rest will have a "mock" configuration that does not use the two-photon laser. The first block will also be the only block that does not have a delay (BKIntv=0). Therefore, the method begins with one baseline scan, followed by a photoactivation scan. The rest of the blocks have a 900 sec BkIntv, and at each time point there are two scans just as at time 0 sec, to maintain the timing consistency.

7. For each of the 10 cells to be followed over time, perform this sequence:
   Find a PAGFP expressing cell-save its imaging method
   Location panel- mark the stage position, specify imaging method
   Main ROI panel-choose a region of interest to be photoactivated Bleach panel-save specific ROI and also load it in the ROI box:
   Erase the ROI and reset the scan zoom to 1
   Find the next cell and set the zoom
   Repeat the process for the next 9 cells

Check that each stage location and all blocks have the appropriate imaging method (scan configuration) associated. Also make sure that the first block at each location corresponds to the appropriate photoactivation method while the rest contain a "mock" method. Finally, select "run".

5. Analyze the PA-GFP Signal Intensity

1. For the cells in which the photoactivatable-GFP signal colocalizes with the red TMRE signal, subtract the background in the PAGFP images (here we use metamorph).
2. Then export the data to excel and calculate the average intensity for z-stacks at each time point. Discard optical sections that have no signal.
3. Measure the signal dilution in each z-stack by calculating the percentage of the original photoactivated signal.

Each data point is scanned and recorded twice, resulting in duplicate z-stack information for each time point. Check that the z-stack values agree. If not; this is indicative of a problem (e.g. focus, cell movement).

6. Representative Results

When a fusion event occurs between an activated and non-activated PA-GFP mitochondrion, the PAGFP within the mitochondrial matrix mixes with the non-labeled matrix and becomes diluted over a larger area, decreasing the signal intensity (**Figure 1A**). In the INS1 cell, a significant decrease in signal intensity occurs every 15 minutes, until equilibration of mitochondrial fusion has been reached (about 1 hour). Note that the cell in **Figure 1B** exhibits nearly complete colocalization of PA-GFP and TMRE signals. In these assays a very low concentration of TMRE (15 nM) is used to help target the photoactivation of PAGFP, and also to monitor cell health. Cells with an abundance of depolarized mitochondria will have incomplete colocalization of PAGFP and TMRE and should not be analyzed because this indicates either phototoxicity, or cells in a dying state.

The equilibration time for mitochondrial fusion is usually 1 hr in INS1 cells, when ~15% of the mitochondrial volume is activated. Sometimes, even if a small area is illuminated, most of mitochondria become photoactivated due to being highly networked, in which case further fusion is difficult to detect. Other cell types may exhibit different equilibration times and should be tested at shorter intervals and over a longer period to characterize mitochondrial dynamics. To inhibit mitochondrial fusion, cells can be placed within a lipotoxic environment. It has previously been demonstrated that 0.4 mM palmitate fragments mitochondria, and inhibits mitochondrial fusion[9]. This effect can be seen in **Figure 2**, where mitochondria are fragmented, but the signal intensity of the mtPAGFP does not change as much as under normal conditions (**Figure 1**). Therefore, the dilution of the PA-GFP signal is likely to be due to mitochondrial fusion rather than fission. In other cells types, we suggest using other known ways to induce mitochondrial fragmentation, such as silencing OPA1 which is necessary for mitochondrial fusion[14].

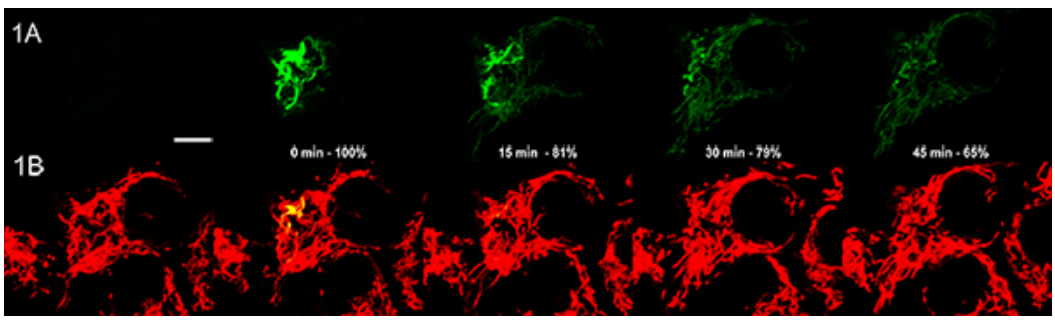

Figure 1. Typical dilution of the PA-GFP signal after photoactivation during a mitochondrial fusion assay. **A.** PA-GFP becomes progressively dimmer due to mitochondrial fusion events leading to the dilution of the protein over an increasing area as can be seen in these projected images of 6 optical sections quantified every 15 minutes. **B.** TMRE with PA-GFP shows that the mitochondria are active and not depolarized. Click here to view larger figure.

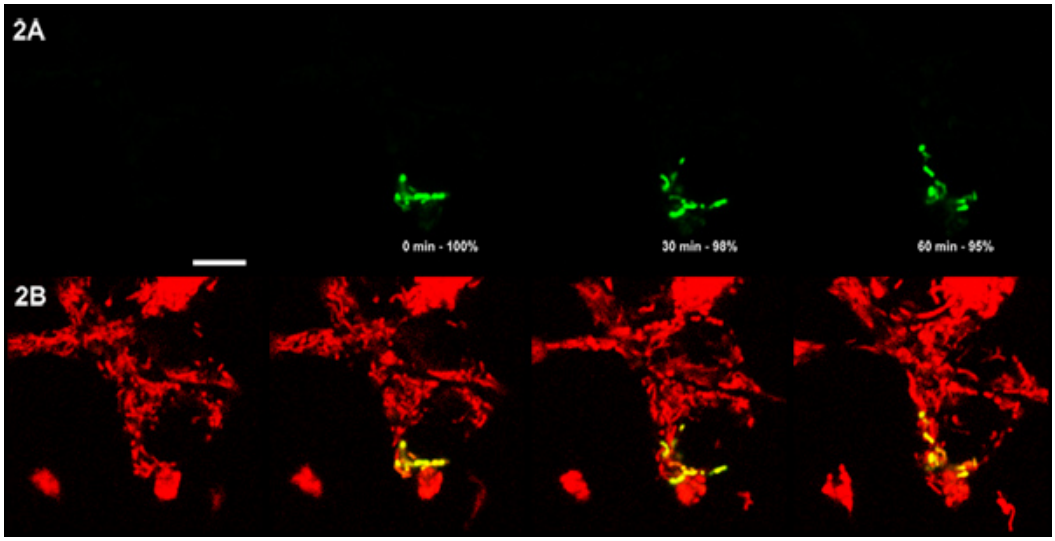

Figure 2. Mitochondrial fusion inhibited with 0.4 mM palmitate decreases the dilution of PA-GFP. **A.** Projections of 6 optical sections showing short mitochondria with an unchanging signal intensity over time. **B.** Colocalization of PA-GFP with TMRE shows that mitochondria are not depolarized. Click here to view larger figure.

Discussion

This method allows for the imaging of around 10 cells at a time, if acquisition occurs every 15 minutes after photoactivation. The exact number of cells will depend on how quickly one is able to locate and mark the mtPAGFP expressing cells within the culture dish, and how quickly one can set up all the software parameters. To make the automation run smoothly an even layer of cells should be used because the designated z-stack margins will apply for all cells.

The size of this initial photoactivation area will govern the equilibration time. To be able to measure mitochondrial fusion, it is important to photoactivate only 10-20% of the network, such that the spread of the signal to the rest of the network can be monitored over time. If too much of the network is photoactivated, it is possible that complete fusion will occur too quickly, and the event will not be captured.

Extreme care must be taken to adjust the laser power of the two-photon laser as well as the TMRE concentration to avoid phototoxicity which leads to mitochondrial depolarization. Ensuring that the mtPAGFP signal colocalizes with the TMRE signal can help in assessing phototoxicity and general cell health [8,15]. Illumination with epifluoerescent light should be avoided. While searching for cells expressing mtPAGFP, the pinhole should be maximally open while scanning with a low power 488nm excitation. Adjusting the two-photon laser power to photoactivate enough PA-GFP to measure the signal over 1 hour but not to oversaturate any of the cells can be tricky [8]. However, time should be spent in this optimization step because once automated program is started, it is tedious to stop it, to choose more cells, and resume.

For quality control, acquisition of a differential interference contrast (DIC) image (or transmitted light) to monitor the focus on the cells can be very helpful and also a good way to detect bubbles formed in the immersion oil during scanning; this sometimes happens from the movements of the stage.

Although using this mtPAGFP method gathers data on the unidirectional movement of mitochondrial matrix proteins from photoactivated mitochondria to those that are not labeled, it is conceivable to utilize this technique to study other processes. For example, specific fluorochromes can be attached to membrane proteins to observe their specific movement during fusion events, as has been shown for ABC-me, the fusion of which occurs on a different time scale from the mixing of soluble matrix proteins [15].

Disclosures

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