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Cell-free Analysis of Mitochondrial Fusion by Fluorescence Microscopy

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Abstract

Dynamin-related proteins on both the mitochondrial outer and inner membranes mediate membrane fusion. Mitochondrial fusion is regulated in many different physiological contexts including cell cycle progression, differentiation pathways, stress responses, and cell death. Mitochondrial fusion is opposed by mitochondrial division and requires movement of mitochondria on microtubules. We developed a cell-free reconstituted mitochondrial fusion assay to circumvent the complexity of the pathways impinging on the activity of the mitochondrial fusion machinery *in vivo*. This allows for quantification of mitochondrial fusion in defined conditions and in the absence of other processes such as mitochondrial division or transport. The impact of proteins or small molecules on mitochondrial fusion can also be assessed. Here we describe the cell-free mitochondrial fusion assay using mitochondria isolated from mouse embryonic fibroblasts.

Keywords

Mitochondrial dynamics; Fusion; Cell-free; Isolated mitochondria; Mitofusin; Opa1; Microscopy

1. Introduction

The state of the mitochondrial network is both a contributor to and indicator of cellular health and function. The overall structure of the network is modulated by the opposing processes of mitochondrial fusion and division, which are both directly controlled by dynamin-related proteins. In mammals, dynamin-related protein 1 (Drp1) is responsible for mitochondrial division, while Mitofusin 1 and Mitofusin 2 (Mfn1 and Mfn2, respectively, Mfns, collectively) and Optic atrophy 1 (Opa1) mediate outer and inner mitochondrial membrane fusion, respectively (1–3). The importance of these processes is highlighted by the finding that loss of any component is embryonic lethal in mouse models (4–8). Additionally, single allele mutant variants of Drp1, Mfn2 and Opa1 are associated with neuropathies (9–12). Therefore, dissecting the mechanisms of mitochondrial dynamics and probing their regulatory pathways are important to understand the fundamental physiology of the cell and the progression of various diseases.

Live or fixed cell imaging of the mitochondrial network using fluorescent markers is widely utilized to query mitochondrial dynamics. While imaging mitochondria in intact cells informs about the overall state of the mitochondrial network under different conditions, this approach has limitations. Live or fixed cell imaging is generally a qualitative description of the network state rather than a quantitative measure of either mitochondrial fusion or division and does not inform on either of these processes individually or over time. Such limitations have been addressed using methods such as fluorescence recovery after photobleaching (FRAP) and photoactivatable fluorescent protein analyses, as recently reviewed in (13). These methods allow assessment of both the degree of connectivity in the mitochondrial network as well network dynamics over time *in situ*. However, analyzing the state of the network as a whole can be complicated by changes in numerous regulatory pathways that impinge on both the division and fusion proteins, thus limiting the ability to draw conclusions solely about a single protein or pathway of interest. Here, we describe in detail a method to quantify mitochondrial fusion in a cell-free assay as first presented in (14). As a reconstituted system, this method measures only mitochondrial fusion and, therefore, is a specific inquiry into the function of the mitochondrial fusion dynamin-related proteins and the proteins that regulate their activity.

In this assay, mitochondria are isolated from two populations of cells stably expressing fluorescent mitochondrial matrix markers of different colors. Isolated, differentially labeled mitochondria are mixed, concentrated by centrifugation, and resuspended in buffer that supports mitochondrial outer and inner membrane fusion. Following visualization by confocal or widefield microscopy, fusion is quantified by comparing the number of fused organelles, the mitochondria with both matrix markers, to the total number of mitochondria. In its simplest form, with only two mitochondrial populations and fusion buffer, the basal rate of mitochondrial fusion from the cells is measured, which allows a direct readout of the activity of the dynamin-related fusion proteins. The assay can then be further exploited to determine the role of other factors including pure protein, crude cytosol or small molecules.

2. Materials

All solutions should be prepared using ultrapure water and analytical grade reagents. Filter sterilization should be performed with 0.2 μ m filters. Dispose of all reagents according to local guidelines.

1. Media appropriate to the cells being used. Complete media for all cells specified in this protocol is: DMEM + high glucose and GlutaMAXTM (Gibco/Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.
2. Plasmids encoding two different mitochondrial matrix targeted fluorescent proteins, such as Addgene #58425 (pclbw-mitoTagRFP) and 58426 (pclbw-mitoCFP) (15).
3. Retroviral packaging cell line Platinum-E (Plat-E) cells (Cell Biolabs, Inc. #RV-101).
4. Mouse embryonic fibroblasts or cells of your choice.

5. Transfection media: we use Opti-MEM™ (Gibco #31985–062).
6. FuGENE™ HD transfection reagent (Promega #E2311).
7. Puromycin and blasticidin antibiotics.
8. Sterile leur lock syringes.
9. 0.45 µm PES membrane syringe filters.
10. Polybrene infection reagent.
11. Freezing media: DMEM + high glucose (no glutamine) supplemented with 20% FBS and 10% tissue culture quality DMSO.
12. Coverslips or glass bottom dishes (such as MatTek #P35G-1.5–20-C).
13. 0.1 M Tris-MOPS pH 7.4: Dissolve 1.21 g Tris powder in about 50 mL water. Adjust the pH of the solution to 7.4 with MOPS powder and bring volume to 100 mL. Filter sterilize and store at 4°C.
14. 1 M sucrose: Dissolve 85.58 g sucrose in water to a total volume of 250 mL. Filter sterilize and store at 4°C.
15. 0.1 M EGTA-Tris: Dissolve 7.62 g EGTA in about 50 mL water. Adjust the pH of the solution to 7.4 with Tris powder and bring the final volume to 100 mL. Filter sterilize and store at room temperature.
16. 3 M sorbitol. Dissolve 136.5 g sorbitol in water to a final volume of 250 mL. Filter sterilize and store at 4°C.
17. 0.5 M PIPES pH 6.8: Dissolve 15.12 g PIPES in about 50 mL water. Adjust the pH of the solution to 6.8 with KOH and bring the final volume to 100 mL (*see* Note 1). Filter sterilize and store at room temperature.
18. 1 M magnesium acetate: Dissolve 10.72 g magnesium acetate in water to a final volume of 50 mL. Filter sterilize and store at room temperature.
19. 5 M potassium acetate: Dissolve 24.54 g potassium acetate in water to a final volume of 50 mL. Filter sterilize and store at room temperature.
20. Mitochondrial Isolation Buffer (MIB): 0.01 M Tris-MOPS (pH 7.4), 0.2 M sucrose, 1 mM EGTA-Tris. Make fresh each day and keep on ice or at 4°C.
21. Cytosol Buffer: 20 mM PIPES pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 0.2 M sorbitol. Make fresh each day and store on ice.
22. 30 mg/mL creatine kinase: Resuspend creatine kinase powder in cytosol buffer at working concentration of 30 mg/mL (*see* Note 2).
23. 1 M creatine phosphate: Dissolve 211.11 mg creatine phosphate in 1 mL cytosol buffer. Store 10 µL aliquots at –80°C.
24. 250 mM GTP: Dissolve 25 mg GTP in 191 µL cytosol buffer. Adjust the pH of the solution to 7.0 with 2 M NaOH (*see* Note 3). Store 5 µL aliquots at –80°C. On the day of the assay, dilute one aliquot to 25 mM with cytosol buffer.

25. 250 mM ATP: Dissolve 25 mg ATP in 197 μ L cytosol buffer. Adjust the pH of the solution to 7.0 with 2 M NaOH (*see* Note 3). Store 5 μ L aliquots at -80°C . On the day of the assay, dilute one aliquot to 25 mM with cytosol buffer.
26. Fusion Buffer: 1.2 mg/ml creatine kinase, 40 mM creatine phosphate, 1.5 mM ATP, 1.5 mM GTP in cytosol buffer.
27. 3% low melt agarose: Dissolve 30 mg low melt agarose in 1 mL cytosol buffer by warming the solution to 90°C . Keep at 65°C .
28. Kontes Potter-Elvehjem tissue grinder, 4 mL (such as Fisher Scientific #K885510-0020).
29. Motor driven homogenizer (such as Caframo #BDC3030).
30. Single well glass slides and covers (such as Fisher Scientific #S175201).
31. Clear nail polish.
32. Fluorescence widefield or confocal microscope.

3. Method

3.1 Expressing fluorescent mitochondrial matrix targeted proteins in mouse embryonic fibroblasts (MEFs)(Figure 1). For all experiments, cells are maintained at 37°C and 5% CO_2 .

1. Day 1: Split Plat-E cells, which have been maintained in complete media supplemented with 1 $\mu\text{g}/\text{mL}$ puromycin and 10 $\mu\text{g}/\text{mL}$ blasticidin, and plate at approximately 80% confluency (approximately 3.5×10^5 cells in each well of a 6 well dish) in 2.5 mL complete media (no antibiotics) (*see* Note 4).
2. Grow Plat-E cells overnight.
3. Day 2: Transfect Plat-E cells with high purity, low endotoxin plasmid DNA (mitochondria-targeted RFP and CFP). Combine 175 μL Opti-MEM with 3 μg of DNA and mix. Add 10 μL of FuGENETM HD directly to the liquid and mix vigorously. Incubate at room temperature for 15 minutes before dropping the mixture onto the Plat-E cells.
4. Incubate Plat-E cells with transfection reagents for 8–24 hours.
5. Day 3: Aspirate all media from Plat-E cells and replace with 2.5 mL complete media. Incubate for 24 hours. At the end of this incubation, you will collect the first virus-containing media for infection.
6. Day 3: 10–24 hours before first infection, plate MEF cells (or cell of your choice) at about 70% confluency (approximately 1×10^5 cells per well of 6 well dish) (*see* Note 4).
7. Day 4: Approximately 48 hours after the transfection of the Plat-E cells, perform first viral infection. Aspirate media from MEF cells and discard. Collect media from Plat-E cells with a sterile syringe. Pass this virus-containing media through a 0.45 μm PES membrane syringe filter onto the MEF cells. Add polybrene

reagent to virus-containing media on the MEF cells at a final concentration of 1 µg/mL. Replace media on Plat-E cells. Incubate for 6–10 hours. Repeat infection procedure and incubate overnight.

8. Day 5: Repeat step 3.1.7 beginning approximately 72 hours after Plat-E cell transfection. Plat-E cells can be discarded after the fourth collection of viral-containing media (*see* Note 5).
9. Day 6: Approximately 96 hours after Plat-E transfection, split the MEF cells and combine duplicate wells into a large dish to expand.
10. Test for expression. Plate some cells on a glass bottom dish or coverslip and image using a fluorescence microscope. For best results in the fusion assay, approximately 95% of the cells need to be expressing the fluorescent protein (*see* Note 6).
11. Freeze several tubes at 2×10^6 cells per mL in freezing media. These can be thawed onto a 15 cm dish and split two days later.

3.2 Growing and harvesting cells

1. Begin cell growth for the assay. Three days in advance of the assay, seed five 15 cm plates with approximately 1×10^6 cells per plate (*see* Notes 7 and 8). To maintain these cells in culture, keep another plate growing (*see* Note 9). Populations needed will vary by experiment but minimally include two populations of wild type cells, each expressing one of the two fluorescent proteins (five plates of matrix RFP and five plates of matrix CFP) (*see* Note 10).
2. On the day of the assay, make 25 mL MIB and store at 4°C or on ice.
3. Working with the five plates for one cell population at a time, remove most of the media from dishes, leaving approximately 5 mL. Detach cells from the plates into the remaining media using a cell scraper (*see* Note 11).
4. Transfer the cells from all five plates for one population to one sterile 50 mL conical tube. Keep tubes on ice while collecting cells from next set of dishes.
5. When all cells have been collected from all cell populations, centrifuge at $300 \times g$ for 10 minutes at 4°C to pellet cells (*see* Note 12).

3.3 Isolation of Mitochondria

1. Return tubes to ice and aspirate media. Finger flick the tube to loosen the cell pellet and then resuspend cells in 5 mL cold MIB.
2. Pellet cells by centrifugation at $300 \times g$ for 5 minutes at 4°C. Aspirate all buffer.
3. Resuspend cells in one pellet volume MIB and transfer to ice cold homogenizers, one for each cell population (*see* Note 13).
4. Homogenize cells on ice with a Teflon pestle operating at 400 rpm. Slowly move the pestle up and down 12–15 times, avoiding the formation of bubbles in the sample by keeping the pestle below the buffer at all times (*see* Note 14).

5. Transfer homogenate to a cold 1.5 mL microcentrifuge tube (Figure 2 Tube A). Rinse the homogenizer with 150 μ L MIB and combine with homogenate in microcentrifuge tube. Spin the homogenate at $300 \times g$ for 5 min at 4°C to remove nuclei and unbroken cells.
6. Transfer the supernatant to a new cold 1.5 mL microcentrifuge (Figure 2 Tube B) (*see* Note 15).
7. Resuspend the cell pellet in one pellet volume cold MIB and put back into the same homogenizer from step 3.2.4.
8. Homogenize on ice as in step 3.2.4.
9. Transfer homogenate to the same 1.5 mL microcentrifuge tube from step 3.2.5 (Figure 2 Tube A).
10. Spin the homogenate at $300 \times g$ for 5 min at 4°C to remove any remaining unbroken cells and nuclei.
11. Combine this supernatant with the supernatant from step 3.2.6 (Figure 2 Tube B). Discard the pellet.
12. Spin the combined supernatant $300 \times g$ for 5 min at 4°C to remove any remaining unbroken cells and nuclei.
13. Transfer the post-nuclear supernatant to a clean, cold 1.5 mL microcentrifuge tube (Figure 2 Tube C). Discard the small pellet from step 3.2.12. Spin the supernatant at $7400 \times g$ for 10 min at 4°C to pellet the mitochondria (*see* Note 16).
14. Transfer the post-mitochondrial supernatant to a cold 1.5 mL microcentrifuge tube and save as the crude cytosol fraction (Figure 2 Tube D) (*see* Note 17).
15. Resuspend the mitochondrial pellet in 1000 μ L MIB and spin at $7400 \times g$ for 10 min at 4°C to wash the mitochondria (Figure 2 Tube C) (*see* Note 18).
16. Aspirate all supernatant. Resuspend the mitochondrial pellet in one pellet volume MIB (*see* Note 19).
17. Determine the mitochondrial protein concentration by Bradford assay (*see* Note 20).

3.4 Fusion assay

1. For each reaction condition, mix 10–15 μ g of mitochondrial protein for each color of mitochondria in a 1.5 mL microcentrifuge tube. Make a master mix in MIB if the same mitochondrial pairing is being tested under multiple conditions and then aliquot mixed mitochondria into individual tubes (100 μ L MIB per reaction in master mix).
2. Pellet the mixed mitochondria by centrifugation at $7400 \times g$ for 10 min at 4°C (*see* Notes 21 and 22).
3. Move the tubes to ice and incubate for 10 min.

4. Remove the supernatant from all tubes.
5. Add 10 μ L of appropriate fusion reaction buffer to each tube on ice to cover the mitochondrial pellet. Fusion buffer as listed in the Materials section is the standard condition. Cytosol buffer can be used as a negative control. Crude cytosol (step 3.3.14) or pure protein can be added to the standard fusion buffer to test the effect of these factors.
6. Resuspend mitochondria in fusion buffer by pipetting up and down, avoiding the formation of bubbles. Leave on ice until all tubes have been resuspended.
7. Incubate at 37°C for 60 min (*see* Note 23).

3.5 Slide preparation and imaging

1. During fusion incubation, prepare slides for imaging.
2. Prepare agarose bed in single well depression slides (Figure 3A): pipet 30–40 μ L hot low melt agarose into the depression (Figure 3B) and immediately put a plain glass slide on top to create a flat surface (Figure 3C) (*see* Note 24). Just before use, move the top slide off of the agarose bed by pushing to the side (do not lift up). Clean off the excess agarose from around the depression with a razor and a delicate task wipe (Figure 3D) (*see* Note 25).
3. At the end of the 60-minute incubation, move tubes to ice.
4. Pipet 4 μ L reaction mixture onto the agarose bed.
5. Drop a coverslip over the reaction mixture.
6. Seal the coverslip with nail polish.
7. Collect images of each reaction with a confocal or widefield fluorescence microscope with the appropriate excitation and emission settings to detect the matrix-targeted fluorophores. Refer to the guidelines below for further detail (*see* Note 26).
8. Collect Z stacks to capture the entire mitochondrial volume in both colors; we recommend 10–12 steps of 0.2 μ m each (*see* Note 27). Collect multiple fields of view from different positions on the agarose bed so that at least 400 total mitochondria are imaged (Figure 4A) (*see* Note 28).
9. Fusion efficiency is quantified by dividing the number of fused mitochondria (those with both fluorophores in three dimensions) by the total number of mitochondria in a given field of view (Figure 4B). Count at least 4 fields and a minimum of 400 total mitochondria.
10. Analyze data by comparing the proportion of fused mitochondria among conditions. Due to daily variation in buffer components, temperature, etc., we normalize fusion efficiency to a control reaction of wild type mitochondria with standard fusion buffer (*see* Note 28).

4 Notes

1. Use KOH pellets until the PIPES goes into solution, then continue adjusting the pH with 6 M KOH.
2. Creatine kinase is a labile enzyme. Aliquot 1–3 mg of powder to microcentrifuge tubes upon receipt and note the weight of the powder on the tube. Store microcentrifuge tubes at 4°C in a desiccator. On the day of the fusion assay, resuspend creatine kinase powder aliquot in cytosol buffer.
3. The pH of GTP and ATP can be measured using pH paper. It is important to adjust the pH of the GTP and ATP to 7.0 to maintain the stability of the triphosphate.
4. We use three wells of a six-well dish for each color; i.e. wild type RFP cells will require three wells of Plat-E cells and three wells of MEF cells.
5. By the fourth collection of viral supernatant, the Plat-E cells will look patchy and begin to lift off the dish.
6. Expression could also be tested and/or selected for by cell sorting.
7. Keep any necessary selection on the cells as they are being maintained and passed. The plasmids that we use for the mitochondrial marker fluorescent proteins do not encode a resistance gene for cell culture. When cells are expanded to five 15 cm dishes, we do not maintain antibiotic selection.
8. Plating these dishes will require one confluent 15 cm dish of cells. The seeding density may need to be adjusted for the cell type to be utilized. On the day of mitochondrial isolation, cells need to be 90 – 100 % confluent.
9. In addition to plates for the fusion assay, we maintain another set of plates for propagation and seeding fusion assay plates.
10. The wild type populations are required every time as controls. Other cell types can be grown as necessary.
11. Cell scrapers can be washed in water then 70% ethanol and used again.
12. Spinning time can be adjusted depending on appearance of the pellet. The pellet should be tight enough so that it does not come off the side of the tube. During this step, rinse homogenizer tubes in water and MIB and leave on ice.
13. About 200–400 μ L.
14. The pestle is inserted into the overhead stirrer and glass tube is held in a small beaker of ice water while performing the strokes. It is imperative that the cells are concentrated at this point.
15. Supernatant should be cloudy.
16. Make cytosol buffer during this step.
17. This supernatant should be clear.

18. This removes any remaining cytosolic factors.
19. The mitochondrial pellet is usually 15 – 30 μ L.
20. Create a standard curve and determine protein concentration according to the manufacturer's instructions. The mitochondrial concentration is usually 2–10 mg/mL.
21. Make working stocks of creatine kinase, creatine phosphatase, GTP and ATP and then fusion buffer during this step.
22. This concentrates the mitochondria to establish proximity.
23. Other time points can be collected as well.
24. It might help to cut off the end of the pipet tip so the viscous agarose is easier to transfer. Make sure there are minimal bubbles in the agarose. Place the top slide before the agarose begins to solidify and at an angle so it can slide off easily (Figure 3C).
25. The slides can be cleaned with 70% ethanol and reused indefinitely.
26. Isolated mitochondria vary in intensity of fluorescent signal, in part because fusion dilutes the fluorophores. When establishing your exposure conditions, ensure that the fluorescent signal is bright enough to visualize all mitochondria, especially those with lower intensity. This may result in some mitochondria with signal near saturation.
27. We use an 100X objective and collect fields of either 512×512 or 1920×1080 pixels.
28. Find fields with enough mitochondria that they are worth counting but not so crowded that you cannot distinguish one organelle from another (as in Figure 4A).
29. Keep raw data along with the proportions that have been normalized to the wild type fusion efficiency.

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Figure 1.
Timeline of generating stable cell lines expressing fluorescent mitochondrial matrix targeted proteins by retroviral production and infection.

Figure 2.
Differential centrifugation overview beginning after homogenization.

Figure 3. Slide preparation schematic. (A) Illustration of single well depression slide. (B) 3% low melt agarose in depression. (C) Plain glass slide placed over the hot agarose creating a flat agarose bed in the depression. (D) Final agarose bed in the depression after the top slide has been removed and extra agarose has been cleaned off.

Figure 4. Example of images of mitochondria after cell-free fusion assay performed in cytosol buffer as a negative control, or fusion buffer. (A) Field of mitochondria obtained at 100X by a widefield fluorescence microscope. (B) Detail of the boxed area in (A) showing mitochondria expressing RFP, CFP, and the merged image. Arrowheads indicate examples of fused mitochondria determined by overlap of RFP and CFP signal. (Scale bar = 1 μ m).