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# Mitoflash lights single mitochondrial dynamics events in mature cardiomyocytes

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ABSTRACT

Mitochondria, the powerhouse of eukaryotic cells, are highly dynamic organelle. Mitochondrial fission, fusion, kissing and contraction have been reported over and over again in non-static cells, such as fibroblast, with tubular mitochondrial networks. Even though the fluorescence propagation among mitochondria of mature cardiomyocytes had been captured using mitochondrial matrix targeted photoactivatable GFP (PAGFP) or MitoDendra proteins, there are no direct evidence that single real time mitochondrial dynamics events exist in mature cardiomyocytes with ball-like mitochondria. Here we first time revealed the visualizable single mitochondrial dynamics events in adult mature cardiomyocytes by the mitochondrial flash (mitoflash). We found fission, fusion, contraction and kissing were accompanied by a mitoflash event. Metabolism could increase mitochondrial contraction. Fusion and Kissing mediated inter-mitochondrial communication with higher frequency than fission. These results demonstrate that mitochondria of static mature cardiomyocytes are undergoing the rare, but real dynamics change.

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## 1. Introduction

Mitochondria are the powerhouse of the eukaryotic cells. They are also participate in free radical production, fatty acid synthesis, Ca2+ homeostasis and cell fate regulation in addition to ATP production [1,2]. In most cells, mitochondria are highly dynamic organelles undergoing constant movement, fusion, fission, kissing and contraction. These processes control not only the shape but also the function of mitochondria [3,4]. Mitochondrial dynamics allows redistribution of mitochondria to bursts of ATP production in response to the ever-changing physiological demands of cells, exchange contents among the mitochondrial population to repair damaged mitochondria [5]. Disruption of mitochondrial dynamics causes a series of diseases, including neurodegenerative disease, cardiovascular disease, and even cancer [6,7]. Mitochondrial dynamics events have been visualized by microscope on non-static cells, such as mouse embryo fibroblast (MEFs), because mitochondrial fission, fusion, movement and contraction were happening frequently in these cells [8–10].

In mature cardiomyocytes, mitochondria as the most prominent and dominant intracellular organelle occupy up to 40% of cell volume. They are highly restricted into a narrow space by myofilaments [11,12]. Thus, mitochondrial movement is highly restricked. They are seem static, neither moving, fusing, nor fission. Nevertheless, the proteins that mediate mitochondrial fusion and fission are highly expressed in hearts [4]. Cardiac mitofusins (Mfn) 1 and 2 ablation induced mitochondrial fragmentation has suggested a putative mitochondrial fission [13,14], dynamin-related protein 1 (Drp1) downregulation induced mitochondrial elongation has also suggested a putative mitochondrial fusion [15].

Several groups have reported the mitochondrial matrix-targeted green PAGFP fluorescence propagation from the 405 nm stimulated area in mature cardiomyocytes. However, the discrepancy has arisen between them. Ether fission and fusion mediate or kissing and nanotunneling mediate inter-mitochondrial communication due to them have not directly catches the single real time mitochondrial dynamics events of mature cardiomyocytes [16–18]. Mitochondrial dynamics event including fission and fusion are more likely to be performed between adjacent mitochondria in mature cardiomyocytes due to their movement limitation [16]. The direct evidence is required to proof the existence of single

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mitochondrial dynamics events in mature cardiomyocytes. The methodology is the main limitation for this discovery. The whole process of mitochondrial fission and fusion is occurring within a tiny restricted site in mature cardiomyocytes. Thus, it is very difficult to catch the mitochondrial fission and fusion events without an activity and obvious readout phenomenon. Mitoflash is a mitochondrial fluorescence transient increase event, which reflects mitochondrial electron transport chain (ETC) activity and metabolism activation, detected by circularly permuted YFP (cpYFP), [19–21]. It amplifies the signal of activated mitochondria and thus made the mitochondria was easily identified from the other static mitochondria. Although the controversies regarding the relative roles of superoxide and pH signals during a flash event was never stop, there was no doubt that mitofash as a biological indicator of activated mitochondria [22–25]. Given mitochondrial fission and fusion are planned to affect the specific mitochondrial matrix components, metabolism and fate through breaking one mitochondria or fusing two mitochondria [3,26], so we thought focusing on mitoflash would potentially give us an opportunity to catch the single mitochondrial dynamics events in mature cardiomyocytes.

## 2. Materials and methods

### 2.1. Recombinant adenovirus vectors

Construction of recombinant adenovirus vectors containing mt-cpYFP (Ad-mt-cpYFP) has been described previously [21]. Ad-mt-cpYFP was amplified in AD293 cells and purified by standard CsCl gradient centrifugation followed by overnight dialysis. The concentration of virus was determined, aliquoted and stored at −80 °C.

### 2.2. Adult cardiac myocytes culture and gene transfer

All animal maintenance and experimental procedures were performed according to Tongji University Guide for the use of laboratory animals. Adult rat cardiac myocytes were isolated from female Sprague Dawley (SD) rats (200–250 g) following the protocol [23]. Briefly, rat was anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital. The heart was quickly removed and cannulated via ascending aorta, and mounted on a modified Langendorff perfusion system. The heart was perfused with 37 °C oxygenated Krebs-Henseleit Buffer (KHB) solution supplemented with collagenase II (80 U/ml, Worthington) and hyaluronidase (0.15 mg/ml, Sigma) for 30 min. The heart was cut into small pieces for further digestion under gentle agitation in enzyme solution. Rod shaped adult cardiac myocytes were collected by brief centrifugation. Myocytes were plated on coverslips (20 mm, round) precoated with laminin (40 μg/ml for 1 h, Invitrogen) at a density of $2–3 \times 10^4$ cells per coverslip and in M199 medium (Sigma) supplemented with 10 mM glutathione, 26.2 mM sodium bicarbonate, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 0.1% insulin-transferrin-selenium-X, 0.02% bovine serum albumin, 50 U/ml penicillin-streptomycin and 5% fetal bovine serum (FBS). Two hours after the plating, the medium was changed to serum-free M199. Freshly isolated mouse myocytes right after 2 h plating were used for confocal imaging. Adenovirus-mediated gene transfer was done at a multiplicity of infection (MOI) of 50–100 on rat cardiac myocytes. The rat myocytes were kept in culture for another 48 h to allow adequate gene expression prior to imaging.

### 2.3. Confocal imaging

Confocal imaging used a Zeiss LSM 510 Meta confocal microscope equipped with a 40 × 1.3NA oil immersion objective and followed a procedure developed previously [22]. Intact myocytes were incubated in KHB buffer at room temperature and in a custom designed perfusion chamber mounted on the confocal microscope stage. Dual excitation images of mt-cpYFP were taken by alternating excitation at 488 nm and collecting emissions at >505 nm. Time-lapse x,y images were acquired at 1024 × 1024 resolution for 100 frames and at a sampling rate of 1 s per frame. Videos were generated using the Zeiss LSM viewer software according to 16 frames per second.

### 2.4. Western blotting

To detect the cpYFP expression in cultured myocytes, 2 days after gene transfer, myocytes were harvested and protein samples were collected in lysis buffer (Cell Signaling). Protein samples were quantified by a BCA kit (novoprotein) and an equal amount of proteins was loaded onto SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membrane and probed with specific antibodies. The antibodies used were: Anti-GFP (1:2000, Roche) and Anti-actin (1:2000, Sigma). Secondary antibodies were conjugated to IRDye 800 (Rockland) or Alexa Fluor 680 (Invitrogen). Signals were visualized and quantified using an Odyssey system (Licor).

### 2.5. Data analysis

Data are presented as mean ± standard error (SE). One way ANOVA, unpaired Student's T test were used when appropriate to determine statistical significance among groups. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Mitochondrial fission in mature cardiomyocyte

We firstly expressed mitochondrial-targeted cpYFP (mt-cpYFP) in cultured adult rat cardiomyocytes using adenovirus mediated gene expression system [20]. The expression of mt-cpYFP was detected using a GFP antibody at 48 h by western blot (Fig. S1A). Myocyte was loaded with tetramethylrhodamine ethyl ester (TMRE), a probe for mitochondrial membrane potential, 30 min in advance before confocal imaging. The cpYFP fluorescence (green) was colocalized with TMRE (red) as seen by the merged image (Fig.S1B) indicated cpYFP was targeted expressing in mitochondria. Even though the mitochondria are seem crowed in mature cardiomyoctyes, most of them are separated by lipid droplets [12] and more independent than the mitochondria of skeletal muscle (Figs. S1C and S1D) [27,28].

We monitored the mitoflash events and caught mitochondrial fission event in cultured mature cardiomyocytes. The fission event was accompanied with a typical mitoflash event. Mother mitochondria underwent contracting, stretching and then splitting to generate two equal daughter mitochondria (Fig. 1A and B, and Video S1). Time course plots showed that the ends fluorescence of the mother mitochondria was different, two daughter mitochondria with slight different fluorescence (Fig. 1C). This consistent with conventional mitochondrial fission to generate two different mitochondria (health and sick) [4]. The events only happen between adjacent mitochondria along myofilament other than Z line (Fig. 1D and E). The whole process completed in 13 ± 2.4 s (Fig. 1F). The fission was occurred at an extremely low frequency. Only 6 events from $6.3 \times 10^6$ mitochondria of 9019 cells in 100 s (Table S1). Base on the average number of mitochondria per myocyte was 706 ± 129. That was approximately 1.0 event per one million mitochondria in 100 s (Table S1).

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