Mitochondrial swelling and restorable fragmentation stimulated by femtosecond laser

Fan Shi,1 Hao He,1,2,∗Yintao Wang,1 Dayong Liu,3 Minglie Hu,1 and Chingyue Wang1

1Ultrafast Laser Laboratory, College of Precision Instrument and Optoelectronics Engineering, Tianjin University, Tianjin, 300072, China
2Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, 200030, China
3Department of Endodontics, School of Stomatology, Tianjin Medical University, Tianjin, 300072, China
∗haohe@tju.edu.cn

Abstract: Mitochondria play a key role in all cellular physiology, processes, and behaviors. It is very difficult to precisely stimulate single mitochondria noninvasively in traditional biomedical research. In this study, we report that femtosecond laser can stimulate fragmentation or swelling of single mitochondria in human mesenchymal stem cells rather than physical disruption or ablation. In experiments, fragmented mitochondria can recover normal very soon but swelling ones cannot. At the same time, laser-induced generation of mitochondrial reactive oxygen species and opening of mitochondria permeability transition pores are involved in mitochondrial responses to photostimulation. Furthermore, the localized translocation of proapoptotic molecules are found in those stimulated mitochondria. Those results suggest femtosecond-laser photostimulation as a noninvasive and precise method for mitochondrial manipulation and related research.

©2015 Optical Society of America

OCIS codes: (170.1530) Cell analysis; (170.7160) Ultrafast technology; (170.1420) Biology.

References and links

1. E. E. Hoover and J. A. Squier, “Advances in multiphoton microscopy technology,” Nat. Photonics. 7(2), 93–101 (2013).
2. S. H. Chung and E. Mazur, “Surgical applications of femtosecond lasers,” J. Biophotonics 2(10), 557–572 (2009).
3. J. Brugués, V. Nuzzo, E. Mazur, and D. J. Needleman, “Nucleation and Transport Organize Microtubules in Metaphase Spindles,” Cell 149(3), 554–564 (2012).
4. A. Heisterkamp, I. Z. Maxwell, E. Mazur, J. M. Underwood, J. A. Nickerson, S. Kumar, and D. E. Ingber, “Pulse energy dependence of subcellular dissection by femtosecond laser pulses,” Opt. Express 13(10), 3690–3696 (2005).
5. W. Watanabe, N. Arakawa, S. Matsunaga, T. Higashi, K. Fukui, K. Isobe, and K. Itoh, “Femtosecond laser disruption of subcellular organelles in a living cell,” Opt. Express 12(18), 4203–4213 (2004).
6. T. Shimada, W. Watanabe, S. Matsunaga, T. Higashi, H. Ishii, K. Fukui, K. Isobe, and K. Itoh, “Intracellular disruption of mitochondria in a living HeLa cell with a 76-MHz femtosecond laser oscillator,” Opt. Express 13(24), 9869–9880 (2005).
7. J. Yoon, S. W. Ryu, S. Lee, and C. Choi, “Cytosolic Irradiation of Femtosecond Laser Induces Mitochondria-dependent Apoptosis-Like Cell Death via Intrinsic Reactive Oxygen Cascades,” Sci. Rep. 5, 8231 (2015).
8. U. K. Tirlapur, K. König, C. Peuckert, R. Krieg, and K.-J. Halbhuber, “Femtosecond Near-Infrared Laser Pulses Elicit Generation of Reactive Oxygen Species in Mammalian Cells Leading to Apoptosis-like Death,” Exp. Cell Res. 263(1), 88–97 (2001).
9. J. Baumgart, K. Kuetemeyer, W. Bintig, A. Ngezahayo, W. Ertmer, H. Lubatschowski, and A. Heisterkamp, “Repetition rate dependency of reactive oxygen species formation during femtosecond laser-based cell surgery,” J. Biomed. Opt. 14(5), 054040 (2009).
10. Y. Sano, W. Watanabe, and S. Matsunaga, “Chromophore-assisted laser inactivation—towards a spatiotemporal-functional analysis of proteins, and the ablation of chromatin, organelle and cell function,” J. Cell Sci. 127(8), 1621–1629 (2014).
11. A. Valle-Prieto and P. A. Conget, “Human Mesenchymal Stem Cells Efficiently Manage Oxidative Stress,” Stem Cells Dev. 19(12), 1885–1893 (2010).
12. A. Wanet, T. Arnould, P. Renard, P. Lou, and N. Peterson, Mitochondrial involvement in stemness and stem cell differentiation, in Cellular Bioenergetics in Health and Disease: New Perspective in Mitochondrial Biology, (Ed. Kerala, 2012), Chap.6.
1. Introduction

Lasers have been making great progresses and providing excellent methods like microscopy for life science. Notably, besides multiphoton microscopy [1], femtosecond lasers are found able to ablate or disrupt cellular organelles precisely in cell surgery. In this case, the average power of femtosecond laser is usually around ten times of it in multiphoton microscopy [2]. In previous reports, cytoskeleton structures like tubulin and actin, and organelles like mitochondria, can be disrupted by femtosecond lasers while partially maintaining viability of cells [3–7] to provide the possibility to study cell behaviors without such cell structures. Here the laser plays a role of “on-off switch” to control the existence of those structures. However, this kind of on-off switch technique is relatively simple, and thus cannot support some special complicated biological studies. In this paper, we report that femtosecond laser can stimulate single mitochondria to a “diseased” state with interesting morphological changes in human mesenchymal stem cells (hMSCs). Laser-initiated mitochondrial reactive oxygen species (mROS) and permeability transition pores take roles in the regulation of mitochondrial responses, followed by interesting localized molecular translocation in the mitochondria [8–10].

Mitochondria in hMSCs can maintain the lower ROS level than it in their mature differentiated progeny for regulation of differentiation and development of hMSCs [11,12]. Therefore, mitochondrial dysfunction is likely to influence the differentiation or gradually initiate complicated developmental dynamics. Currently, the role and actual functions of mitochondria in hMSCs can be only investigated by biochemical treatment to cells which then stimulate mitochondrial damage indirectly and invasively [13,14]. Such biochemical stimulation is lack of controllability and spatial/temporal resolution. Besides, some unknown cellular processes can be also activated simultaneously.

2. Materials and methods

Experimental setup and preparation

Femtosecond laser technology brings possibility of noninvasive, precise, direct, and controllable methodology for mitochondrial study in stem cells. Here, as a demonstration, a Ti: Sapphire laser at 810 nm (repetition rate of 80 MHz and pulse duration 75 fs) is utilized for photostimulation to single mitochondria in hMSCs. The output power was 400 mW and the laser beam was at first expanded to match the back aperture of a high N.A. objective (60X, water immersed, N.A. = 1.2) of a confocal microscope (Olympus FV1000/IX81, inverted) to get a diffraction-limited focus (diameter~0.9 μm). The vertical focus length was around 2.1 μm. The irradiance could be around 6 × 10^5 W/cm^2 for laser irradiation (peak irradiation~10^11 W/cm^2 in each pulse) at 5 mW. The nonlinear effect of femtosecond laser can be confined in the focal volume with little thermal diffusion by this high N.A. objective. The femtosecond laser was at first expanded and collimated by a lens pair, and then coupled into this microscope by setting a dichroic mirror (transparent at visible but reflective at near
infrared range) between the objective and the beam splitter mirror under it. The mitochondria could be then observed at real time after the photostimulation. The photostimulation, controlled by a mechanical shutter, could be performed during confocal microscopy to observe cell responses at real time. The optical setup was shown in Fig. 1.

![Optical setup based on a confocal system. One more laser at 543 nm was also used in this setup for red fluorescence excitation. A lens pair (Lens 1 and 2) was used for beam expansion, collimation, and divergence control of the femtosecond laser. DM1: reflection < 750 nm, transmission > 750 nm. DM2: transmission at only 488/543/632 nm, and reflective at all other wavelengths. BS: beam splitter.](image)

In experiments, hMSCs were acquired from human dental marrow (experiments exfoliated human deciduous teeth were performed with the permission of the Ethical Committee of Tianjin Medical University) seeded in 35 mm petri dishes with a 0.17-mm glass slide (Nest) for confocal microscopy, and cultured with Dulbecco’s modified Eagle’s medium containing 10% Fetal Bovine Serum (FBS), 20 μM L-glutamine, and 1% (v/v) penicillin/streptomycin at 37 °C with 5% CO₂. Cells were loaded with MitoTracker Green (from Life Technology, final concentration: 300 nM) to label mitochondria, which was tested and compared with cells transfected with mitoGFp for photobleaching and fluorescence stability. In this study, since photostimulation and mitochondrial responses were very fast, no significant photobleaching or fluorescent self-decay was observed. A targeted spot from a single mitochondrial tubule was selected randomly and moved to the focus of femtosecond laser by tuning the microscope stage. Since the laser focus diameter was smaller than 1 μm, it could be confirmed a single mitochondrion was stimulated. To be simple, in this study, a straight continuous mitochondrial (typical tubule structure) was treated as a single one in following description. It should be noted that the laser focus might be vertically apart from the microscopy plane since the femtosecond laser and visible continuous-wave lasers for fluorescence excitation did not share the same optical path and beam shape. Then the vertical position of the femtosecond laser focus was corrected by tuning the divergence angle of the beam by changing the distance of the lens pair for collimation and beam expansion as our previous work [15].

### 3. Results and discussion

#### 3.1 Mitochondrial fragmentation and swelling by photostimulation

To provide moderate stimulation to mitochondria, the femtosecond laser power was decreased to around 6 mW at specimen (peak irradiance~1.1 × 10¹¹ W/cm²), very close to the typical power level in two-photon microscopy. The targeted mitochondrion was stimulated for 100 ms. Immediately after photostimulation, the mitochondria showed no changes. There was neither ablation of the mitochondrion, nor any other responses in the photostimulated mitochondrion. But after several seconds, the stimulated mitochondrion might soon turn
fragmented, as shown in Fig. 2(a) (n = 9 fragmented mitochondria in 102 stimulated ones). Interestingly, the fragmented mitochondrion recovered after another tens of seconds. Some other stimulated mitochondria turned swelling and could never recover back as shown in Fig. 2(b) (n = 8 swelling mitochondria in 102 stimulated ones). Therefore, we treated swelling as a worse diseased response than fragmentation. The rest of stimulated mitochondria did not show any response to photostimulation. If they were stimulated by higher laser power, the rate of fragmented and swelling mitochondria would increase as shown in Fig. 2(c). In experiments, the fragmentation could take place in the whole stimulated mitochondrion (69.23%, n = 65 fragmented mitochondria) after photostimulation, like a propagation. There was only one fragmentation in the rest mitochondria (30.77%). It should be noted that all those fragmentations and swellings were only observed in photostimulated mitochondria. As a control, mitochondria labeled with MitoTracker Green was directly tested with two-photon microscopy by high-intensity (~8 mW) femtosecond laser for 30 minutes. No photobleaching was observed.

We then showed that the mitochondrial response was dependent on different photostimulation powers and durations. As shown in Fig. 3, generally, along with the increase of laser power and stimulation duration, the total rate of fragmented and swelling mitochondria rose. If the laser power was increased to 17 mW (peak irradiance~3.4 × 10^{11} W/cm^2) and stimulation duration 3 s (Fig. 3(d)), all mitochondria in the stimulated cell would be swelling. Besides, it should be noted that the stimulation effect could not be determined simply by the incident laser energy (power multiplied by stimulation duration). For example, the rate of damaged mitochondria in Fig. 3(d) by 17 mW for 0.3 s (5.1 mJ) was similar to the result in Fig. 3(a) by 8 mW for 3 s (24 mJ). The damage rate in the group stimulated by 10 mW for 0.3 s (3 mJ, Fig. 3(b)) was even less than it in groups by 12 mW (peak irradiance ~2.2 × 10^{11} W/cm^2) and 17 mW for 0.1 s (1.2 mJ and 1.7 mW in Fig. 3(c) and 3(d)). As a control, we used a continuous-wave (CW) laser at 810 nm to stimulate mitochondria in the same way, but with higher power (30 mW). There was no responses in the stimulated mitochondria (n = 30). Therefore, both the multiphoton excitation and thermal effect by the laser may contribute to the stimulation.
3.2 Role of mROS and mPTP

We suspected that the laser-initiated ROS generation, the key point in the classic photodamage mechanism [15], contributed to photostimulated mitochondrial response [16]. To verify this hypothesis, the ROS level in the stimulated mitochondria was measured. To present significant and typical phenomena, mitochondria were then stimulated by the femtosecond laser at 17 mW for 0.1 s. It could be found in Fig. 4(a) that the mitochondrial ROS increase (mROS, assayed by dihydrorohdamine 123 from Life Technology) was initiated by photostimulation while the mitochondrial membrane potential (MMP, assayed by tetramethylrhodamine, methyl ester from Life Technology) was significantly depolarized (n = 5). There was no ROS generation in other mitochondria or in the cell. Therefore, mROS generation might participate in mitochondrial fragmentation and swelling. To further prove this point, mROS was then scavenged by MitoTEMPO (5 μM, from Life Technology) or enhanced by tert-butyl hydroperoxide (TBHP, 100 μM from Sigma) before photostimulation. It could be found from Fig. 4(b) that the rate of mitochondrial morphological change was significantly decreased with MitoTEMPO or increased with TBHP at 6 mW, 10 mW, and 17 mW for 0.1 s respectively (p-value: 0.015, 0.05, 0.0001 by Fisher’s exact test respectively, by comparing the MitoTEMPO and TBHP group). Therefore, those results suggested that mROS generation contributed to mitochondrial fragmentation and swelling.
We then further studied the dynamics of mitochondrial permeability transition pores (mPTP) who regulated MMP depolarization by their opening [17]. The state of mPTP was assayed by mPTP kit (from Life Technology, indicated by the fluorescence intensity of calcein which could be quenched by Co²⁺ diffusing from cytosol into mitochondria if mPTP were open). As shown in Fig. 4(c), the fast decline of calcein fluorescence in the stimulated mitochondrion suggested the opening of mPTP after photostimulation at 17 mW for 100 ms (n = 5). Interestingly, if mPTP were inhibited by cyclosporine A (CsA, 1 µM), the photodamage effect would be enhanced (p = 0.0046, Fisher’s exact test), as shown in Fig. 4(d) (at 6 mW for 0.1 s, n = 60). Therefore, the laser-induced mROS did not damage mitochondria through mPTP, but the transient mPTP opening might help to release mROS to cytosol to prevent mitochondria from photodamage.

3.3 Localized translocation of bax and cytochrome c induced by photostimulation

The mPTP opening could induce translocation of Bax, a member of Bcl-2 family that would concentrate onto mitochondria in the whole cell when apoptosis started [18,19]. We therefore studied the localization of Bax after photostimulation as demonstration for application of this optical method for mitochondrial research. By immunofluorescent microscopy, Bax concentrating merely onto the stimulated mitochondria could be found in Fig. 5(a). Subsequently, the release of cytochrome C from the stimulated mitochondria modulated by Bax was also found as shown in Fig. 5(b). The cytochrome C would then provide more oxidative stress to the mitochondria for more stimulation. Different from classic apoptosis processes, such “gentle” photostimulation that induced localized translocation of Bax did not initiate apoptosis when tested after 6 hours by trypan blue and propidium iodide. Therefore, this precise photostimulation provides the possibility to research apoptosis at single organelle level but in a “live” cell.
4. Conclusion

In conclusion, we report here that weak femtosecond-laser photostimulation can induce interesting morphological change of mitochondria in hMSCs instead of simple ablation or disruption of them. Fragmentation and swelling of mitochondria can be excited by such “gentle” stimulation and the fragmented mitochondria can soon recover. This photostimulation has a very high spatial and temporal resolution (submicron and 0.1 s level), and the single-shot one-spot stimulation provides little influence to other parts of the whole cell. The laser-generated mROS are involved in mitochondrial damage and mPTP also play a role in this process. The mPTP opening will lead a localized translocation of proapoptosis molecules in the stimulated mitochondria, which, different from classic apoptosis mechanism, does not initiate cell apoptosis. All those biological responses are mostly localized inside or around the stimulated single mitochondrion. Similar results could be acquired in HeLa and HpeG2 cells. Our results thus provide an optical method to induce moderate and noninvasive stimulation to mitochondria for research of mitochondria in apoptosis, ageing, and related fields in cell biology and mitochondrial disease [20].

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81571719 and 61322502), Open Project of State Key Laboratory of Modern Optical Instrumentation, Zhejiang University, and Program for Changjiang Scholars and Innovative Research Team in University (IRT13033).