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TITLE:

Photostimulation by Femtosecond Laser Activates Extracellular-Signal-Regulated Kinase (ERK) Signaling or Mitochondrial Events in Target Cells

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SUMMARY:

Tightly-focused femtosecond laser can deliver precise stimulation to cells by being coupled into a confocal microscopy enabling the real-time observation and photostimulation. The photostimulation can activate cell molecular events including ERK signaling pathway and mitochondrial flashes of reactive oxygen species.

ABSTRACT:

Direct control of cellular defined molecular events is important to life science. Recently, studies have demonstrated that femtosecond laser stimulation can simultaneously activate multiple cellular molecular signaling pathways. In this protocol, we show that through coupling femtosecond laser into a confocal microscope, cells can be stimulated precisely by the tightly-focused laser. Some molecular processes that can be simultaneously observed are subsequently activated. We present detailed protocols of the photostimulation to activate extracellular signal regulated kinase (ERK) signaling pathway in Hela cells. Mitochondrial flashes of reactive oxygen species (ROS) and other mitochondrial events can be also stimulated if focusing the femtosecond laser pulse on a certain mitochondrial tubular structure. This protocol includes pretreating cells before photostimulation, delivering the photostimulation by a femtosecond laser flash onto the target, and observing/identifying molecular changes afterwards. This protocol represents an all-optical tool for related biological researches.

INTRODUCTION:

The technology of controlling cellular signaling molecules is an important part of the development of life science. Traditionally, the most commonly-used method is biochemical treatment by drugs or biological materials[1-3]. Over the past decade, the invention of

optogenetics opens a new era for cellular molecular signal modulation. Transfection with light sensitive proteins by gene engineering makes light become a powerful tool to modulate various protein activities in target cell. This technology has made encouraging progresses such as excitation and inhibition of neural signal, promoting gene expression, manipulating cellular signal patterns, leading different cell fates and pathological investigation[4-9]. However, light can only work by transfecting cells with optogenetic proteins. In current stage, there exist rare methods that enable light to control cellular molecules directly besides optogenetics.

Femtosecond laser has advanced biological researches by providing efficient multiphoton excitation while maintaining good biological safety. By deploying diverse photo processing strategies, it has realized numerous achievements such as multiphoton microscopy, microsurgeries and multiphoton optogenetic applications[10-16]. Recent investigations show that femtosecond laser stimulation has been demonstrated as a highly efficient optical method to directly induce molecular signaling events. It has been found that tightly-focused femtosecond laser irradiation on endoplasmic reticulum (ER) is able to deplete calcium in ER and activate calcium-release-activated calcium (CRAC) channels to form calcium signals in cells[17]. This photo-activated calcium signal can spread between multiple types of cells[18-20]. Furthermore, it also has the ability to activate cell signaling pathways such as nuclear factor of activated T cells (NFAT) and ERK signaling pathway[21, 22]. By adjusting the intensity and localization of femtosecond laser exposure in cells, for example, focusing the laser on mitochondria, it can influence mitochondrial morphology and molecular events[23-25]. Specifically, bursts of mitochondrial ROS generation can be excited by photostimulation, which is remarked as fluorescent flashes in mitochondria (mitoflashes).

Hence, the photostimulation technology is of good potential to be widely applied in related biological research. It is also a good chance to extend femtosecond laser applications in controlling of cellular signaling molecules and functions besides microscopy. Here, we provide the technical details of photostimulation. The photostimulation is achieved by coupling a femtosecond laser to a confocal microscope to provide single target cell with a short flash photostimulation. It can initiate efficient and controllable ERK activation in the cell. If the photostimulation is located on the mitochondrial tubular structure, the mitochondrial membrane potential, morphology, ROS, and permeability transition pores, can all be controlled by the photostimulation. Based on this photostimulation scheme, we provide a detailed method of activating ERK signaling pathway and influencing multiple mitochondrial events in Hela cells. This protocol elucidates the process of delivering femtosecond laser stimulation into target cells.

The photostimulation system is established on a confocal microscope with a femtosecond laser coupling into it for simultaneous stimulation and continuous microscopy. The femtosecond laser (wavelength: 1040 nm, repetition rate: 50 MHz, pulse width: 120 fs, maximum output average power: 1 W) is split into two beams before coupling. One is guided through a relay telescope consisting of a pair of lenses. It is then directly reflected into the back-aperture of an objective (60X, N.A. = 1.2, water immersion) to form a diffraction-limit focus (Stim-A). The other is reflected to the scanning optical path of confocal microscope to work as a two-photon

scanning mode (Stim-B). Stim-A presents a fixed focus point in the center of field of view (FOV). Stim-B is a pre-designed partial confocal scanning area in the FOV. Stim-A and Stim-B are shown in **Figure 1A**. A CCD camera under the dichroic mirror (DM) provides bright-field imaging for monitoring the focus of femtosecond laser.

There are some crucial essentials for the following experiments. In this protocol, a femtosecond fiber laser source (1040 nm, 50 MHz, 120 fs) is used as an example. In practice, most commercial femtosecond oscillators can be used as long as the pulse width is shorter than 200 fs and the peak power density should be above the level of 10^{11} ~ 10^{12} W/cm^2. For example, a Ti: Sapphire laser usually used for multiphoton microscopy is able to replace the femtosecond laser showed in **Figure 1B**. The laser power and some other photostimulation parameters need to be tuned because the optical parameters (pulse width, wavelength, and repetition rate) vary a lot in different femtosecond lasers that thus induce different multiphoton excitation efficiencies.

Along with femtosecond laser stimulation, the confocal microscopy provides continuous cell imaging to monitor molecular dynamics in real time in both Stim-A and Stim-B modes. Both photostimulation schemes (Stim-A and Stim-B) are controlled by a mechanical shutter with millisecond resolution (**Figure 1**).

In Stim-A mode, the position of laser focus is fixed in the center of FOV. A relay telescope is used to ensure the focus of femtosecond laser to be located on confocal imaging plane by tuning the distance between two lenses in the vertical direction (the laser propagation direction, vertical to the confocal imaging plane, as shown in **Figure 1**). By the bright-field imaging of CCD camera, the diameter of laser focus can be measured (~2 μm, **Figure 2B**). The stimulation durations and exposure times are controlled by a shutter during the confocal imaging process.

In Stim-B mode, the stimulation area can be pre-assigned manually in the confocal imaging controlling software to any form like line, polygon, or circle. The shutter is synchronized with the confocal scanning process. It opens at the pre-designed time which is set through the confocal imaging controlling software. Then, the stimulation area is scanned by femtosecond laser as confocal microscopy. Thus, the sample is only stimulated by femtosecond laser when confocal scanning process enters a given imaging frame.

The photostimulation system can be established on both inverted and upright metallurgical microscopes according to the experiment subjects. In vitro cells cultured in Petri dishes are better to work with inverted microscopes. Animals, especially brains of live animals, are more suitable with upright microscopes. In this study, we take the inverted microscope as an example. It should be noted that the cover of Petri dish is not opened during the whole experiments.

PROTOCOL:

CAUTION: The protocol presented below involves using NIR femtosecond laser and toxic chemicals. Please pay attention to all possible damages induced by experiment procedures. Please read safety data sheets of all relevant chemicals or other materials before use. Please

follow the safety instructions of the laser facilities or consult professionals for guidance before operate laser source.

1. Experimental preparation

1.1. Setting up the photostimulation system

NOTE: The system consists of a femtosecond laser and a laser (at visible range for fluorescence excitation) scanning confocal microscope. In **Figure 1**, a fiber femtosecond laser (1040 nm, 50 MHz, 120 fs, 1 W) is used for coupling. The parameters are not fixed or necessary. It can be replaced by a Ti: Sapphire laser or other commercial femtosecond oscillators in the NIR range.

1.1.1. Tune on the femtosecond laser and the confocal microscope.

NOTE: Please set femtosecond laser power at a low level (~ 50 mW) in the process of adjusting the optical path.

1.1.2. Use reflective mirrors (RM1 and RM2 showed in **Figure 1B**) to direct femtosecond laser beam through a mechanical shutter. Open the shutter.

1.1.3. Set a 50/50 beam splitter (BS, **Figure 1B**) to split the femtosecond laser beam into two separate beams (transmission beam and reflection beam). Steer RM2 and BS to make the femtosecond laser beam coincide with the scanning laser beam.

NOTE: (1) A reference iris (Iris 1, **Figure 1B**) behind the long-pass dichroic mirror (DM, 700 nm cut-on wavelength, **Figure 1B**) is used for positioning scanning laser path. (2) The system can only work at Stim-A or Stim-B mode, respectively. For Stim-A mode, the BS can be removed. For Stim-B, the BS can be replaced by a RM.

1.1.4. Set a relay telescope consisting of a pair of lenses to expand the transmission beam width, which is consist with the back aperture of the objective.

NOTE: (1) The reference iris 2 and 3 (**Figure 1B**) are set to collimate the femtosecond laser beam (**Figure 1B**). (2) The magnification of the relay telescope depends on the original diameter of femtosecond laser beam and the diameter of back aperture of the objective.

1.1.5. Steer RMs (RM 3-5, **Figure 1B**) to align the expanded beam into microscope. Steer RM 4 and RM 5 to tune the focus of femtosecond laser to the center of FOV.

1.1.6. Measure the transmission efficiency of the objective of the femtosecond laser.

NOTE: Please measure the laser power at Stim-A and Stim-B respectively due to the different penetration paths of the laser in those two modes. It will use the power at specimen to illustrate related experiment procedures below.

1.1.7. Tune off the shutter, femtosecond laser and confocal microscope until experiments start.

NOTE: In the following experiments, Stim-A and Stim-B do not work together. If using Stim-A, the femtosecond laser beam of Stim-B needs to be blocked. On the other hand, the beam of Stim-A should be blocked if the system works at Stim-B mode.

1.2. Prepare the culture medium: Dulbecco's modified Eagle's medium (DMEM) high glucose with 10% fetal bovine serum (FBS).

1.3. Prepare the ERK2-GFP DNA plasmid, Mito-GFP DNA plasmid and mitochondrial matrix-targeting circularly permuted yellow fluorescent protein (mt-cpYFP) DNA plasmid. Keep the plasmids at -20 °C until use.

1.4. Prepare tetramethylrhodamine (TMRM, 100 μM), and polyethylenimine (PEI 1 mg/mL). Keep them at -20 °C until use.

1.5. Prepare 5% paraformaldehyde, 0.5% Triton X-100, anti-eIF4E (eukaryotic translation initiation factor 4E), antibody (phosphor S209, 1 mg/mL), anti-Bax antibody (1 mg/mL), anti-cytochrome C antibody (1 mg/mL), secondary antibody (anti-Rabbit IgG H&L, Alexa Fluor 488, 1 mg/mL), and 1% BSA with 0.1% Tween20. Keep these reagents at 4 °C until use.

1.5. Prepare sterile materials: cell culture bottles, Petri dishes with glass slide bottom (**Figure 3A**), dishes with a glass bottom, an imprinted 500 μm cell location grid (**Figure 3B**, to localize the photostimulated cells), 1.5 mL and 10 mL tubes, and 1 mL, 100 μL and 10 μL pipettes and tips.

1.6. Prepare standard cell culture laboratory equipment: a cell culture incubator set at 5% CO_2 and 37 °C, and a biological safety cabinet.

2. Cell culture and transfection

NOTE: Hela cell (cell line derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks)[26] is used as an example in this protocol.

2.1. Cell passage

2.1.1. Remove the culture medium from cell culture bottle containing enough cells.

2.1.2. Wash the bottle with 2 mL of phosphate-buffered saline (PBS). Remove the PBS.

2.1.3. Add 1 mL of trypsin slowly and tap the bottle slightly. Put the bottle back in the incubator and incubate the cells 3 min at 37 °C.

2.1.4. Remove the trypsin. Add 2-3 mL of culture medium. Pipette the medium several times to help the cells to detach. Transfer the medium with cells to a 10 mL tube.

2.1.5. Take 10 µL of the sample from the tube for cell counting.

2.1.6. Seed ~25,000 cells into a Petri dish (**Figure 3A**) and add culture medium up to 1 mL.

2.1.7. Put the dish contained cells back in the incubator. Incubate the cells 24 h at 37 °C before transfection.

2.2. Cell transfection

2.2.1. Use 0.5 µg of DNA plasmid (ERK2-GFP, Mito-GFP or mt-cpYFP) for transfection per dish. Add 0.5 µg of plasmid and 2.5 µg of PEI into 50 µL of DMEM in a 1.5 mL tube. Incubate mixed media 10 min at room temperature.

2.2.2. Take the dish with the cells from incubator. Replace culture medium with 1 mL of DMEM.

2.2.3. Add DNA/PEI mixed media into the cells drop by drop. Put the cells back in the incubator.

2.2.4. Incubate the cells 3 h at 37 °C and replace DMEM DNA/PEI mixed media with 1 mL of culture medium.

2.2.5. Incubate transfected cells 24 h at 37 °C before photostimulation experiments.

3. Activation of ERK2 by photostimulation of femtosecond laser.

3.1. Turn on femtosecond laser and ensure the shutter is closed.

3.2. Turn on the laser-scanning confocal microscope and open the microscope software. Set the excitation laser at 488 nm. Set the power level of 488 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels. Set interval time of each pixel as 2.4 µs. Set the interval time between two frames at 6 s to minimize photobleaching and photodamage to cells. Set total imaging frames at around 300 frames to provide ~30-min continuous microscopy in an individual experiment.

NOTE: The interval of two adjacent frames, total imaging frames and imaging time of continuous microscopy can be adjusted according to experimental requirements. If an individual experiment lasts over 2 h, please set the CO_2 incubation system (shown in **Figure 4**) for microscope as present in step 3.3 to provide the environment of 5% CO_2 and 37 °C for maintaining cell viability. If an individual experiment is limited within 2 h, the CO_2 incubation system is not necessary.

3.3. Turn on the CO_2 incubation system, turn on all heater and set working temperature at 37 °C. Wait until the temperature goes up to 37 °C and concentration of CO_2 up to 5%. Put the incubator stage on the microscope stage as shown in **Figure 4A**.

3.4. Prepare Hela cells transfected with ERK2-GFP as described in step 2.

NOTE: In an individual experiment, the scheme Stim-A or Stim-B is applied to deliver photostimulation to cells. Step 3.5 and 3.6 present detailed steps under Stim-A and Stim-B respectively.

3.5. Delivering femtosecond laser stimulation into target cells using Stim-A mode

NOTE: Before photostimulation procedure, please ensure that the diameter of the focus is around 2 μm. This process is described in step 3.5.1 and 3.5.2.

3.5.1. Take the dish containing cells transfected with ERK2-GFP from incubator and put the dish on the microscope stage.

3.5.2. Start fast scanning mode through the microscope operating software. Tune the objective to acquire clear fluorescent images of cells. Stop fast scanning. Switch to bright-field imaging mode by the CCD camera (**Figure 2A**). Open the shutter and adjust the distance between two lenses of the relay telescope to ensure the diameter of femtosecond laser focus to be ~ 2 μm (**Figure 2B**). Close the shutter to complete adjustment.

NOTE: A reference arrow can be labeled to indicate the laser focus (**Figure 2**). In the meantime, a reference arrow can also set at the center of the fluorescent imaging window to indicate the position of the focus of femtosecond laser.

3.5.3. Set the power of femtosecond laser at 15-40 mW (810 nm, 65 fs, 80 MHz), or 20-60 mW (1040 nm, 120 fs, 50 MHz) at the specimen. Set opening time of the shutter at 0.05-0.2 s.

3.5.4. Use fast scanning mode to select a target cell well expressed ERK2-GFP.

3.5.5. Move the stage in order to localize the cytosol area of the selected target cell at the center of FOV under bright-field imaging of the CCD camera (**Figure 2A**).

3.5.6. Click the **Start** bottom to start continuous microscopy imaging progress.

3.5.7. Open the shutter at any pre-defined time slot to deliver the femtosecond laser stimulation designed in step 3.5.3 into the target cell.

NOTE: (1) The photostimulation can be performed at any time in the confocal microscopy sequence controlled by the shutter. (2) The photostimulation can be delivered for multiple times in the same position during the confocal microscopy sequence.

3.5.8. Wait until the imaging process is complete. Save the imaging data for further data analysis.

3.6. Delivering femtosecond laser stimulation into target cells using Stim-B mode

3.6.1. Set the power of femtosecond laser at 15-40 mW (810 nm), 20-60 mW (1040 nm) at the specimen.

3.6.2. Take the dish containing cells transfected with ERK2-GFP from the incubator and put it on the microscope stage.

3.6.3. Use fast scanning mode to select a target cell well expressed ERK2-GFP.

3.6.4. Set the confocal imaging process as step 3.2. Define a special scanning frame as the stimulation frame in the imaging process. Define the parameter of the simulation frame.

3.6.4.1. Set a scanning region (2 × 2-3 × 3 μm^2) at the cytosol area close to the nucleus in the target cell.

3.6.4.2. Set total scanning time at 0.1-0.2 s. Synchronize the shutter of femtosecond laser with the confocal scanning according to the predefined photostimulation area, which is only open when the laser scanning drops in the stimulation frame and close immediately when it goes out.

NOTE: (1) The photostimulaion region can be set to any size. (2) The photostimulation can be performed at any predefined time slot in the confocal microscopy sequence. (3) The photostimulation can be performed for multiple times on the same or different pre-defined areas in the FOV in that confocal microscopy sequence.

3.6.5. Click the **Start** bottom to start continuous microscopy imaging progress.

3.6.7. Wait until the imaging process is complete. Save the imaging data for further data analysis.

3.7. Turn off the femtosecond laser and confocal microscope after the experiment.

4. Activation of eIF4E (substrate of ERK) by femtosecond laser simulation

4.1. Hela cell preparation

4.1.1. Follow steps 2.1.1-2.1.5.

4.1.2. Seed ~20,000 cells into a Petri dish with cell location grids (**Figure 3B**) to localize the photostimulated cells. Add culture medium up to 1 mL.

4.1.3. Put the dish with the cells back in the incubator. Incubate the cells 24 h at 37 °C before femtosecond laser treatment.

4.2. Turn on the femtosecond laser and ensure the shutter is closed.

4.3. Turn on the laser-scanning confocal microscope and open the microscope software.

4.4. Prepare the CO_2 incubation system as statement in step 3.3.

4.5. Delivering femtosecond laser stimulation into target cells using Stim-A mode

4.5.1. Set the power of femtosecond laser at 15-40 mW (810 nm), 20-60 mW (1040 nm) at the specimen. Set opening time of the shutter at 0.05-0.2 s.

4.5.2. Take the dish with cells from the incubator and mount it in the CO_2 incubator on the stage of microscope.

4.5.3. Move the objective in vertical direction to locate the imaging plane under bright-field imaging of the CCD camera. Move the specimen stage in horizontal direction to ensure that no cell locates in the center of FOV. Open the shutter and adjust the distance between two lenses of the relay telescope to ensure the diameter of femtosecond laser focus at ~2 μm. Close the shutter to complete adjustment.

4.5.4. Move the microscope stage to randomly select 5~10 grids. It can be indicated and localized by the grids in the bottom of the Petri dishes under bright-field imaging of the CCD camera. Mark/record the coordinates of selected grids in the dish.

4.5.5. Stimulate all cells located in the selected grids one by one manually under bright-field imaging of the CCD camera.

4.6. Put the dish back in the incubator. Incubate the cells 24 h at 37 °C before immunofluorescence microscopy. Turn off the femtosecond laser and confocal microscope after the photostimualtion procedure.

4.7. Immunofluorescence microscopy of phosphorylated eIF4E in cells with photostimulation for the confirmation of ERK activation

4.7.1. Take the dish containing cells with photostimulation treatment in step 4.5 out of the incubator. Remove the culture medium. Wash the cells with PBS once. Remove PBS.

4.7.2. Add 1 mL of 5% paraformaldehyde (4 °C) into the dish. Fix the cells with 5% paraformaldehyde for 10 min. Remove the paraformaldehyde buffer. Wash the cells with PBS for 5 min twice. Remove PBS.

4.7.3. Add 1 mL of 0.5% Triton X-100 into the dish. Incubate the cells 15 min at room temperature. Remove the Triton X-100 buffer. Wash the cells with PBS for 5 min twice. Remove PBS.

4.7.4. Add 1 mL of 1% BSA buffer into the dish. Incubate the cells 30 min at room temperature. Remove the BSA buffer.

4.7.5. Dilute anti-eIF4E antibody (phosphor S209) in 1 mL of PBS with 1% BSA to a final concentration of 1 μg/mL. Add the buffer into the dish. Incubate the cells for 10-12 h at 4 °C. Remove the buffer.

4.7.6. Wash the cells with PBS for 5 min twice. Remove PBS.

4.7.7. Dilute the secondary antibody anti-Rabbit IgG H&L in 1 mL of PBS with 1% BSA to a final concentration of 2 μg/mL. Add the buffer into the dish. Incubate the cells 2 h at room temperature. Remove the buffer.

4.7.8. Wash the cells with PBS. Remove PBS.

4.7.9. Add 1 mL of PBS into the dish.

4.7.10. Turn on the laser scanning confocal microscope. Open the microscope software. Set excitation laser at 488 nm. Set the power level of 488 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels. Set interval time of each pixel as 2.4 μs.

4.7.11. Put the dish with immunofluorescent staining cells on the microscope stage. Locate the selected boxes under bright-field imaging of the CCD camera.

4.7.12. Start single frame confocal scanning. Save the fluorescent pictures of photostimulation cells.

4.7.13. Move the stage randomly to locate areas with no femtosecond laser stimulation. Start single frame confocal scanning. Save the fluorescent pictures of no stimulation cells as control data.

4.8. Turn off the confocal microscope after the experiment.

5. Activation of mitoflashes and other mitochondrial events by photostimulation.

NOTE: To observe mitochondrial morphological dynamics, Hela cells are transfected with Mito-GFP in step 5.1 to fluorescently indicate mitochondria. To observe mitoflashes, Hela cells are transfected with mt-cpYFP in step 5.1.

5.1. Prepare Hela cells transfected with Mito-GFP or mt-cpYFP following step 2.

5.2. Turn on femtosecond laser and ensure the shutter is closed.

5.3. Turn on the laser-scanning confocal microscope. Set excitation laser at 488 nm. Set the power level of 488 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels. Set total imaging time of each frame as 2.2 s. Set the interval time between two frames at 0 s. Set total imaging frames as 200 frames to provide ~ 440 s continuous microscopy in an individual experiment.

NOTE: The interval of two adjacent frames, total imaging frames and imaging time of continuous microscopy can be adjusted according to experimental requirements.

5.4. Prepare the CO_2 incubation system as described in step 3.3 if needed.

5.5. Delivering femtosecond laser stimulation into the target mitochondrion using Stim-A mode

5.5.1. Take the dish containing cells transfected with Mito-GFP or mt-cpYFP from incubator and put the dish on the microscope stage.

5.5.2. Check the status of femtosecond laser as step 3.5.2. Ensure that the focus of femtosecond laser is located in the center of FOV. Set a reference arrow at the center of the fluorescent imaging window to indicate the position of the focus.

5.5.3. Set the power of femtosecond laser at 5-30 mW (810 nm), 10-40 mW (1040 nm) at the specimen. Set opening time of the shutter at 0.05-0.1 s.

5.5.4. Use fast scanning mode to select a target cell well expressed Mito-GFP or mt-cpYFP.

5.5.5. Select one mitochondrion randomly in the target cell as the experimental subject. Move the microscope stage in order to localize the target mitochondrial tubular structure at the center of FOV (indicate by the reference arrow) by fast scanning mode.

5.5.6. Click the **Start** bottom to start continuous microscopy imaging progress.

5.5.7. Open the shutter at any pre-defined time to deliver the femtosecond laser stimulation designed in step 5.5.3 into the target mitochondrial tubular structure.

NOTE: (1) The femtosecond laser exposure on the mitochondrial tubular structure can be controlled by the shutter at any time during the confocal microscopy. (2) Perform only one femtosecond-laser exposure in one experiment because one femtosecond laser stimulation may perturb the mitochondrial status over a long period.

5.5.8. Wait until the imaging process is complete. Save the imaging data for further data

analysis.

5.6. Turn off the femtosecond laser and confocal microscope after the experiment.

6. Oscillation of mitochondrial membrane potential on target mitochondria in Hela cells by femtosecond laser stimulation.

6.1. Prepare Hela cells following the steps 2.1.1-2.1.6.

6.2. Incubate the cells for 24 h at 37 °C before experiment.

6.3. Prepare the TMRM staining solution: dilute the TMRM in 1 mL of DMEM with 10% FBS to a final concentration of 100 nM.

6.4. Take the dish with cells prepared in step 5.2.1 and 5.2.2 out of the incubator. Remove the culture medium. Add the TMRM staining solution prepared in step 6.3 into the dish. Stain for 15-20 min at 37 °C in the incubator. Remove the staining solution. Wash the cells with PBS once. Add 1 mL of culture medium into the dish.

6.5. Turn on femtosecond laser and ensure the shutter is closed.

6.6. Turn on the laser-scanning confocal microscope. Open the microscope software. Set excitation laser at 532 nm. Set the power level of 532 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels and frame generation time at 2.2 s. Set the interval time between two frames at 0 s. Set total imaging frames as 200 frames to provide ~440 s continuous microscopy in an individual experiment.

NOTE: The interval of two adjacent frames, total imaging frames and imaging time of continuous microscopy can be adjusted according to experimental requirements.

6.7. Prepare the CO_2 incubation system described in step 3.3 if needed.

6.8. Use Stim-A mode to deliver femtosecond laser stimulation into the target mitochondrion. Follow steps 5.5.1-5.5.8 to complete experiment.

NOTE: In step 6.8, select a mitochondrion which is well stained with TMRM as the target mitochondrion.

6.9. Turn off the femtosecond laser and confocal microscope after the experiment.

7. Changes of Bax and cytochrome C on the target mitochondria in Hela cells by femtosecond laser stimulation.

NOTE: In this experiment, seed the cells in Petri dishes with cell location grids (**Figure 3B**) to

localize the cell which is treated by femtosecond laser. Stain the cells with TMRM to localize the mitochondrion which is selected to be stimulated by femtosecond laser.

7.1. Prepare Hela cells in a Petri dish with cell location grids (**Figure 3B**) as described in step 4.1.

7.2. Stain the cells with TMRM as described in steps 6.3 and 6.4.

7.3. Turn on femtosecond laser and ensure the shutter is closed.

7.4. Turn on the laser-scanning confocal microscope. Open the microscope software. Set excitation laser at 532 nm. Set the power level of 532 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels and frame generation time at 2.2 s. Set the interval time between two frames at 0 s. Set total imaging frames as 50 frames to provide ∼110 s continuous microscopy in an individual experiment.

7.5. Delivering femtosecond laser stimulation into the target mitochondrion using Stim-A mode

7.5.1. Set the power of femtosecond laser at 5-30 mW (810 nm), 10-40 mW (1040 nm) at the specimen. Set opening time of the shutter at 0.05-0.1 s.

7.5.2. Take the dish containing cells stained with from the incubator and put the dish on the microscope stage.

7.5.3. Use fast scanning mode to select the target cell which is well stained with TMRM.

7.5.4. Move the stage in order to localize the target mitochondrial tubular structure at the center of FOV by using fast scanning mode. Mark the coordinate of the grid which the selected cell is located under bright-field imaging.

7.5.5. Click the **Start** bottom to start continuous microscopy imaging progress.

7.5.6. Open the shutter at any pre-defined time of microscopy imaging progress manually to deliver the femtosecond laser stimulation designed in step 7.5.1 into the target mitochondrial tubular structure.

7.5.7. Wait until the imaging process is complete. Mark the selected mitochondrion which is simulated by femtosecond laser. Save the imaging data for further data analysis.

7.5.8. Turn off the femtosecond laser and confocal microscope after the experiment. Prepare immunofluorescence microscopy of Bax or cytochrome C on the experiment cells.

7.6. Immunofluorescence microscopy of Bax or cytochrome C on the femtosecond laser

treated mitochondrion.

7.6.1. Take the dish from the microscope stage.

7.6.2. Complete immunofluorescent staining of Bax or cytochrome C follow steps 4.7.1-4.7.9.

NOTE: Use anti-Bax or anti-cytochrome C instead of anti-eIF4E in step 4.7.5 to finish immunofluorescent staining of Bax or cytochrome C in step 7.6.2.

7.6.3. Turn on the laser-scanning confocal microscope and open the microscope software. Set excitation laser at 488 nm and 532 nm. Set the power level of 488 nm and 532 nm laser at 0.1 mW. Set imaging size as 512 × 512 pixels and frame generation time at 2.2 s.

7.6.4. Put the dish with immunofluorescent staining cells on the microscope stage. Locate the selected grid under bright-field imaging of the CCD camera. Locate the cell which is treated by femtosecond laser.

7.6.5. Start single frame confocal scanning. Locate the mitochondrion which is stimulated by femtosecond laser. Save the fluorescent picture of photostimulation cell for further data analysis.

7.7. Turn off the confocal microscope after the experiment.

REPRESENTATIVE RESULTS:

The photostimulation can be performed simultaneously along with continuous confocal scanning microscopy. The photostimulation can start at any pre-defined time slot in the time-lapse confocal microscopy sequence. The confocal microscopy can monitor cellular molecules by fluorescent imaging. The molecular responses to photostimulation and other dynamics can be identified in this way. Theoretically, if ERK is activated, it will be phosphorylated move from the cytoplasm to cell nucleus[27]. The specific cell fate can be regulated by the certain patterns of this ERK signal[28]. In recent studies, optical modulation based on optogentics has provided a high precise control of the ERK signal in duration and magnitude and revealed that perturbed ERK signal transmission dynamics drives improper proliferation in cancer cells[7,8]. Here, we demonstrate the ERK2 translocation into nucleus after treating cell with a short flash of femtosecond laser by using the method presented in this protocol. As shown in **Figure 5A**, ERK2-GFP fluorescence reaches the maximum after several minutes of femtosecond laser simulation. The ERK2 molecules will be dephosphorylated after activating downstream substrates in the nucleus, and then the ERK2 comes back to the cytoplasm indicated by decreasing of nuclear GFP fluorescence. ERK2 can be activated for multiple times by multiple photostimulations (**Figure 5B**). Therefore, it is able to manipulate the ERK2 signal pattern precisely by controlling interval time between multiple stimuli. In addition, ERK2 can be activated in adjacent cells around the stimulated cell occasionally (**Figure 5C**). This observation indicates that some diffusible molecules may be released by the cell treated with femtosecond laser to activate ERK2 in the adjacent cells. Phosphorylation of ERK2 downstream protein eIF4E

can be confirmed and visualized by immunofluorescence microscopy (**Figure 5D**). This result indicates that femtosecond laser stimulation can successfully activate ERK signaling pathway. More detailed results are in Wang S., et al.[22].

Mitochondrial oxidative flashes (mitoflashes) are oxidative bursts in mitochondria that root from complex mitochondrial molecular dynamics. In last past decade, mitoflashes are realized to be an elemental mitochondria signaling event and take an important part in multitudinous cell functions[29-31]. Traditionally, mitoflashes are usually observed by chance when treating cells with chemicals to provide indirect stress to mitochondria[29,30]. By implementing this photostimulation scheme, we achieve a controllable and precise manner to excite mitoflashes at single mitochondrial tubular level. The successful mitoflash excitation is shown in **Figure 6A**. Interestingly, the properties of mitoflashes such as pulse peak, width and response durations[32] are closely related to the femtosecond laser power. More detailed quantitative analysis of mitoflashes excited by femtosecond laser stimulation is in Wang S., et al.[32]. This photostimulation to mitochondria also shows varieties of mitochondrial molecular dynamics, including fragmentation and restoration of mitochondrial morphology (**Figure 6B**), and oscillation of mitochondrial membrane potential (**Figure 6C**). Similar to photo-activated mitoflashes, these mitochondria events have different performance with different power intensities of photostimulation. It is different from activation of ERK signaling pathway. The influence of femtosecond laser is highly restricted on a single mitochondrial tube. More detailed results are in Wang Y., et al.[24] and Shi F., et al.[25].

FIGURE AND TABLE LEGENDS:

Figure 1: The photostimulation scheme established on a femtosecond laser coupling into a confocal microscope. (A) Optical paths of (B) the photostimulation and confocal imaging system. The femtosecond laser is at first split by a 50/50 beam splitter into two beams. The transmission is expanded by a relay telescope and then reflected into the objective to form Stim-A. The reflection beam is aligned through the microscope scanning system to form Stim-B. A CCD camera is used to provide a bright-felid imaging to monitor cells and focus of femtosecond laser in Stim-A mode. Stim-A: a fixed focus in the center of FOV; Stim-B: a special scanning frame at pre-designed area. DM: dichroic mirror, BS: beam splitter, RM: reflective mirror. The wavelength of confocal scanning laser is 488 nm/532 nm/635 nm, and the typical collection wavelength interval of fluorescence is < 560 nm/560-625 nm/> 625 nm. The fiber femtosecond laser (1040 nm, 120 fs, 50 MHz, 1 W) can be replaced by a Ti: Sapphire laser (810 nm, 80 MHz, 65 fs, 1 W) or other commercial femtosecond laser oscillators.

Figure 2: In Stim-A mode, the localization and size of femtosecond laser focus on the target cell at confocal imaging plane under bright-felid imaging. (A) Femtosecond laser is blocked by the shutter. (B) Femtosecond laser is on. Arrow: the reference arrow is set at the center of FOV to confirm that the femtosecond laser focus locates the correct position. Bar: 10 μm.

Figure 3: The Petri dish used for cell culture, transfection and photostimulation experiments. (A) The 35-mm Petri dish with a 15-mm diameter and 0.17-mm thickness glass bottom. (B) The 35-mm Petri dish with a glass bottom and an imprinted 500 μm cell location grid.

Figure 4: The CO_2 incubation system. (A) The CO_2 incubator stage and (B) the control panel.

Figure 5: ERK photoactivation by using Stim-A mode. (A) Single photo stimulus (1040 nm, 40 mW, 0.1 s) induces ERK2-GFP translocation into nucleus and then back to cytoplasm. (B) ERK2 signal pattern mediated by single femtosecond laser exposure (red arrow, 1040 nm, 40 mW, 0.1 s) and two photostimulations (green arrows, 810 nm, 30 mW, 0.1 s). (C) ERK activation in surrounding cells by single short femtosecond laser exposure in target cell (white arrow, 810 nm, 24 mW, 0.2 s). (D) The Immunofluorescence of eIF4E-P shows a significant increase 24 h after single photosimulation (810 nm, 25 mW, 0.2 s). Bar: (A) 10 µm, (B) and (C) 20 µm. The fluorescence of ERK2-GFP and eIF4E-P is excited by 488 nm excitation laser and collected in < 560 nm channel.

Figure 6: Multiple mitochondria events induced by photostimulation under Stim-A mode. (A) Excitation of mitoflash on the stimulated mitochondrial tubule by single stimulus (810 nm, 16 mW, 0.1 s). (B) Mitochondria morphological fragmentation and restoration induced by single photostimulation (1040 nm, 30 mW, 0.1 s). (C) Oscillation of mitochondrial membrane potential by single femtosecond laser stimulation (1040 nm, 20 mW, 0.1 s). Arrows in (A) (B) and (C) indicate the position of photostimulations. Bar: 10 µm. The fluorescence of Mito-GFP or mt-cpYFP is excited by 488 nm excitation laser and collected in < 560 nm channel. The fluorescence of TMRM is excited by 532 nm excitation laser and collected in 560-625 nm channel.

Table 1. Recommended stimulation duration and average power of femtosecond laser in Stim-A mode.

Table 2. Recommended stimulation duration and average power of femtosecond laser in Stim-A mode at 2 × 2-3 × 3 μm^2 in cell.

DISCUSSION:

We demonstrate a photostimulation strategy by combining a femtosecond laser with a laser scanning confocal microscope system. The photostimulation can directly work as a two-photon microscopy by defining Stim-B accordingly. We provide a detailed protocol for utilizing a short flash of femtosecond laser to trigger ERK signaling or mitoflashes in target cells. The different stimulation modes can be performed according to different experimental purposes and systems. Stim-A can be easily set up based on a confocal microscope. The femtosecond laser can be focused at diffraction-limit level at the FOV center. The subcellular target needs to be moved to the femtosecond laser focus position manually and can be stimulated for arbitrary durations, totally independent from confocal microscopy. Therefore, Stim-A can protect the sample that is out of the focus area perfectly and is suitable for long-duration photostimulation. The stimulation region of Stim-B can be pre-defined as any area in the FOV. It can be performed automatically. But the stimulation duration and exposure time can only follow along with the confocal scanning. After setting the dwell time of each pixel and the total frame size, the stimulation duration in a single microscopy frame is actually fixed. The photostimulation can also be performed periodically frame by frame. It can also be used to activate photosensitizers

and optogenetic proteins that requires less exposure duration but large area.

A critical consideration in this protocol is how to determine the parameters of femtosecond laser stimulation. According to our previous studies, the cellular molecular activity is mostly induced by multiphoton exaction[17,21-25]. Generally multiphoton exaction efficiency is related with all femtosecond laser parameters including wavelength, pulse width, repetition rate. The cellular response is further related with the laser power, stimulation position/region, and exposure duration simultaneously. In principle, the stimulation efficiency conflicts with cell safety. Stronger photostimulation such as higher average power, longer stimulation duration and larger total incident laser energy contributes to higher stimulation efficiencies but brings higher risks of cell damage. Since those parameters are also relative with each other and all contribute to the stimulation and damage effect, it is impossible to use only one parameter, like the total incident laser energy, or laser average power, to define it is safe or not. Considering both signal activation efficiency and cell safety, here, we provide recommended average power and stimulation duration of two femtosecond laser source for reference in **Table 1** and **Table 2**. Besides, the parameters of femtosecond laser stimulation should be changed according to the actual optical system and biological sample.

According to the discussions above, we intend to present more detailed effective ranges of photostimulation parameters. The wavelength of femtosecond laser can be changed at NIR range used for photostimulation. The pulse width should be short to provide a high peak power and high nonlinear exaction efficiency. Long pulse duration is not recommended for this protocol. Usually, the typical pulse width for two-photon microscopy (< 200 fs) is the proper choice. The repetition rate of the laser is not a key factor. The repetition rate up to MHz is suitable for this protocol. The most critical factor for cellular molecular responses is the laser power and stimulation duration of femtosecond laser. For activating ERK, according to our previous work[22], the average power at 15 mW (810 nm) or 20 mW (1040 nm) and exposure duration at 0.2 s are enough to provide sufficient stimulation to activate ERK signaling while maintaining ultrahigh cell viability in Hela cells. On the contrary, average power over 80 mW (810 nm) or 120 mW (1040 nm) and exposure duration longer than 1 s can induce irreversible damage to cells. Mitochondria are generally more sensitive to photostimulation. The stimulation with an average power higher than 40 mW (810 nm) or 80 mW (1040 nm) and exposure duration over 0.2 s may induce significant damage like irreversible fragmentation in stimulated mitochondrion or even in all mitochondria across the whole cell. It should be noted that the photosensitivity of mitochondria to photostimulation varies a lot in different cell types. For example, in HeLa cells, the laser power needs only around 6 mW (810 nm, 0.1 s duration). All mitochondria can response to the photostimulation in the form of fragmentation, mitoflashes, and MMP oscillations. But in human mesenchymal stem cells, the power needs to be increased to around 15 mW (810 nm, 0.1 s duration), and still around half stimulated mitochondria show no response to photostimulation. In Stim-B mode, another factor may affect the photo activation efficiency and cell damage is the stimulation area. The photostimulation may cause cell damage by delivering on a small stimulation area. But the same stimulation may fail to activate ERK by delivering on a larger stimulation area. We recommend that the stimulation area in target cell is around 2×2 - 3×3 μm^2 and does not

exceed 25 μm^2.

The localization of stimulation region is also important for applying this protocol. According to previous works[17,22], the photostimulation in that area can deplete the Ca^{2+} store in ER effectively and activate the CRAC channel. Then, the Ca^{2+} influx can activate ERK signaling pathway subsequently. Therefore, we recommend delivering the photostimulation on the ER region in Hela cells to achieve a high ERK activation efficiency. The ER region can be easily distinguished from cytoplasm or nucleus under bright-field microscopy by a phase-contrast objective. In order to introduce mitochondrial signaling events, the focus of femtosecond laser can be easily mounted on the selected mitochondrial structure following the related procedures.

Photostimulation methods described in this protocol are also able to be used to affect other multiple cellular events such as inducing Ca^{2+} and ROS signals[17,20,21]. It should be noted all those previous works have provided the evaluation of cell safety after photostimulation. For example, significant morphological changes of cells, bubbling, mitochondrial fragmentation and swelling of the whole cell, decreased proliferation rate of cells, and some other unusual changes of the fluorescence during confocal microscopy all imply high cell damage. It should be very careful to monitor the cell status. Nevertheless, with well control of the laser power and stimulation parameters, the cell viability could be maintained in a very high level simultaneously with high stimulation efficiency. Hence, this photostimualtion method of femtosecond laser is of good potential to further extend to more areas and applied in relevant applications.

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DISCLOSURES:

The authors declare no competing interests.

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Figure 1

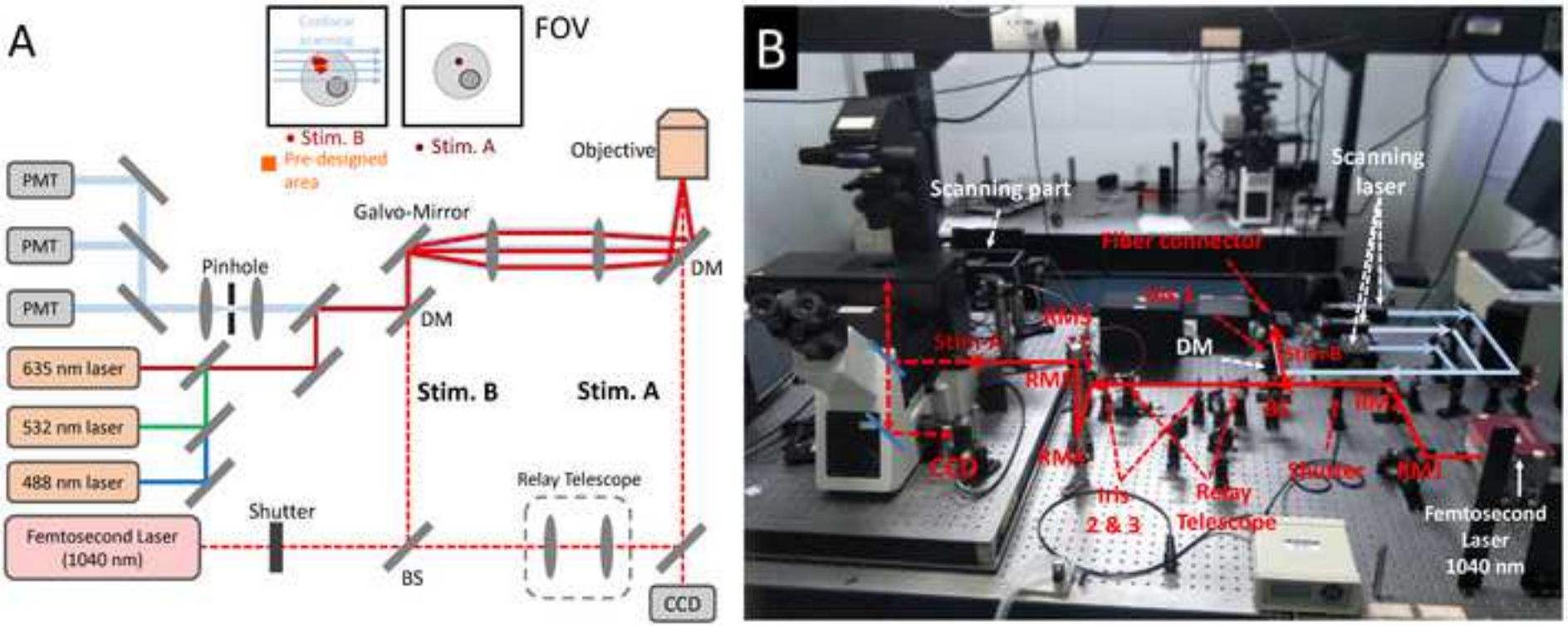

Figure
2

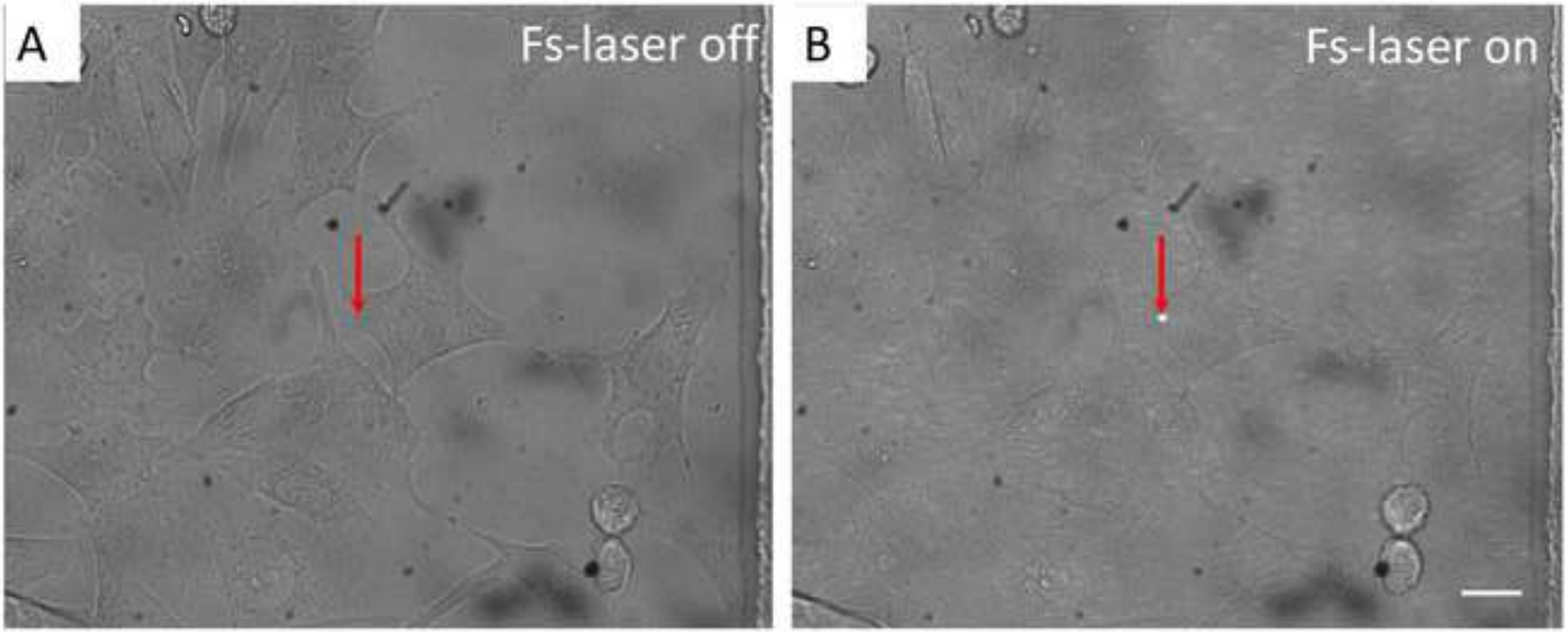

Figure 3

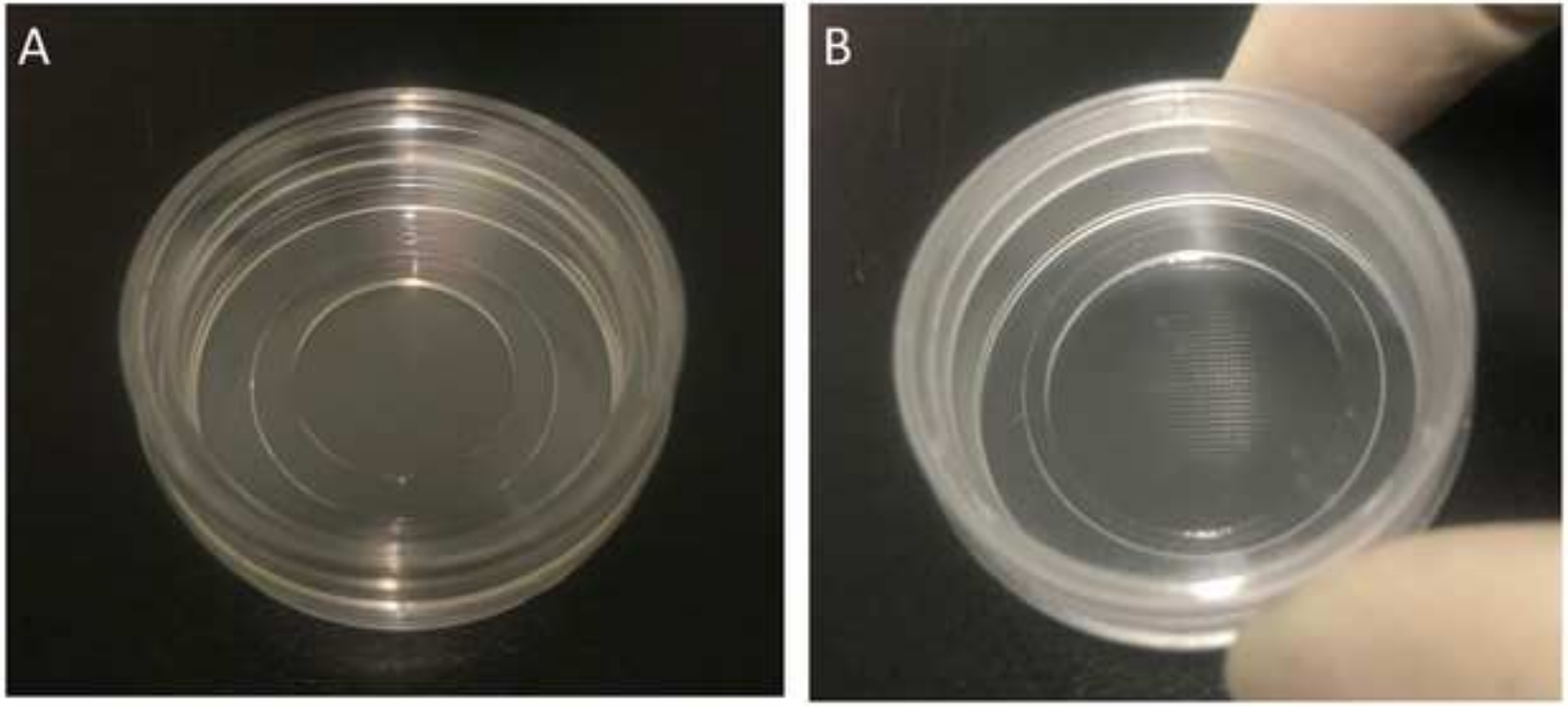

Figure 4

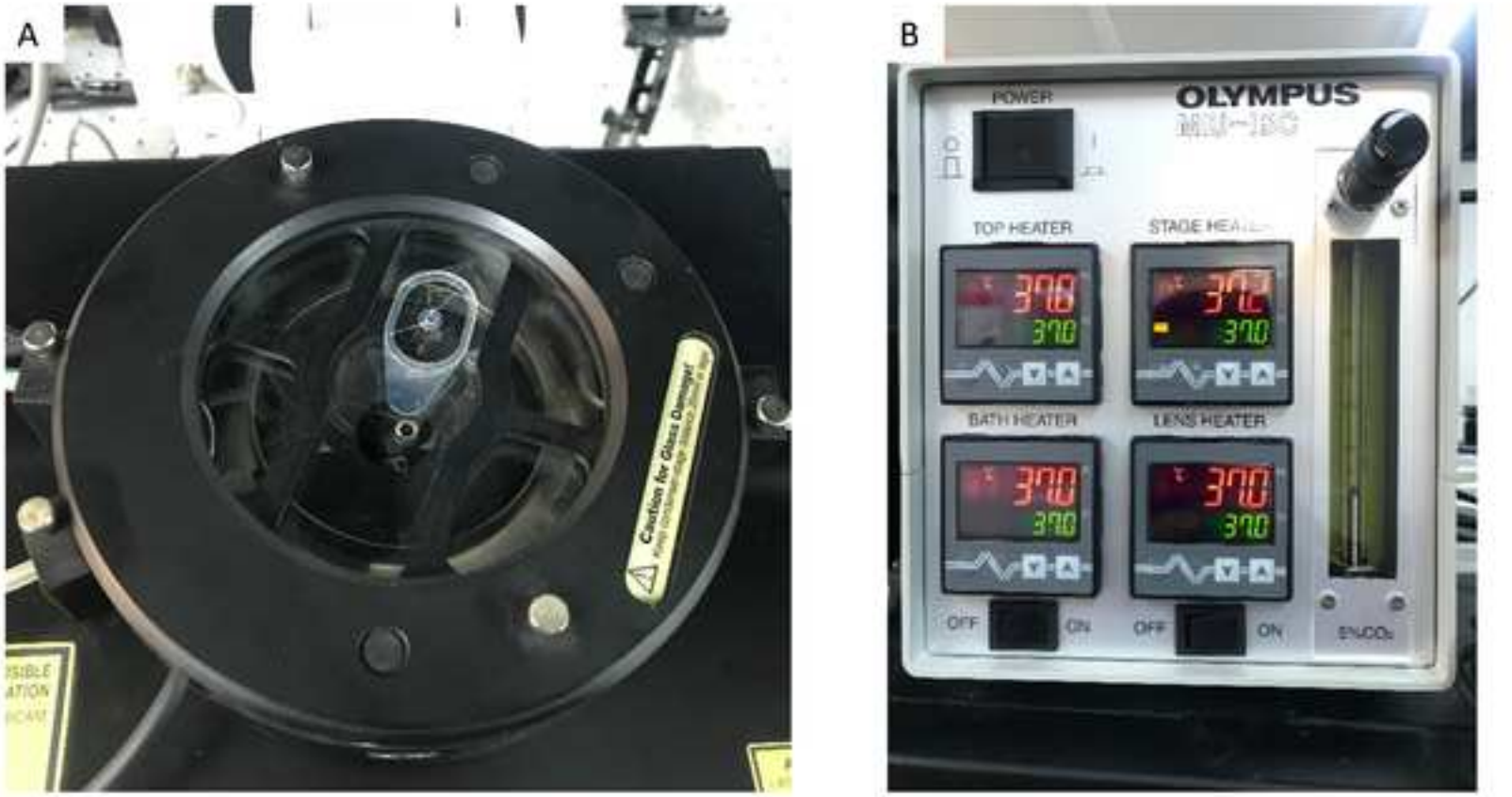

Figure 5

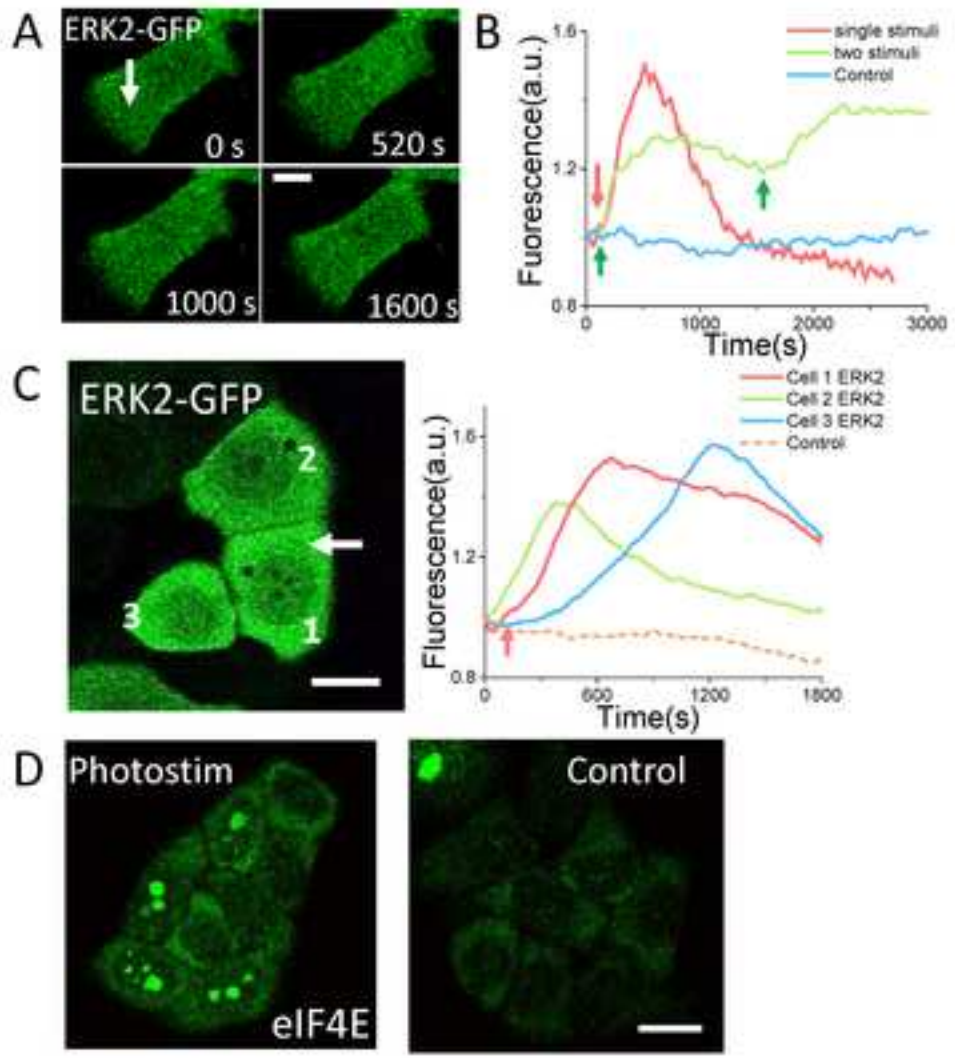

Figure 6

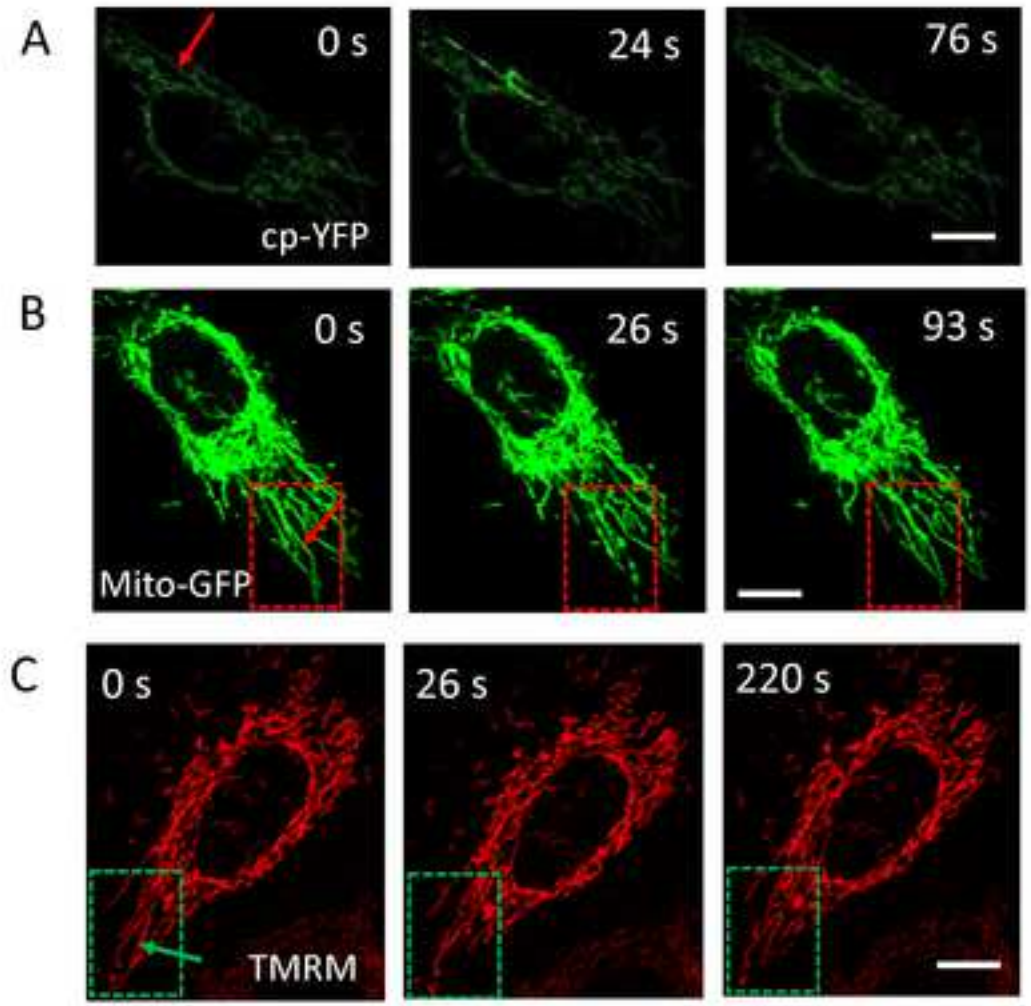

Table 1. Recommended stimulation duration and average power of femtosecond laser in Stim-A

	0.05 s	0.1 s	0.2 s
810 nm, 65 fs, 80 MHz	20 - 65 mW	10 - 60 mW	5 - 50 mW
1040 nm, 120 fs, 50 MHz	30 - 100 mW	20 - 80 mW	15 - 70 mW

A mode

0.5 s

5 - 40 mW

10 - 60 mW

Table 2. Recommended stimulation duration and average power of fs-laser in Stim-B mode a

	0.05 s	0.1 s	0.2 s
810 nm, 65 fs, 80 MHz	25 - 40 mW	20 - 30 mW	15 - 25 mW
1040 nm, 120 fs, 50 MHz	40 - 60 mW	30 - 50 mW	25 - 40 mW

t 4 - 10 µm^2 area in cell

0.5 s

10 - 25 mW

20 - 30 mW

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
inverted microscope	Olympus		
femtosecond laser	Fianium		
CO2 incubation system	Olympus	MIU-IBC	
petri dish	NEST	801002	
petri dish with imprinted grid	Ibidi	81148	
ERK-GFP	addgene	37145	A gift from Rony Seger's lab
mt-cpYFP			A gift from Heping Cheng's lab
mito-GFP	Invitrogen	C10508	
Tetramethylrhodamine (TMRM)	Invitrogen	T668	Dilute in DMSO
polyethylenimine (PEI)	Sigma-Aldrich	9002-98-6	Dilute in PBS
paraformaldehyde	Solarbio	P8430	Dilute in PBS
Triton X-100	Solarbio	T8200	Dilute in PBS
Bovine Serum Albumin (BSA)	Sigma-Aldrich	9048-46-8	Dilute in PBS
Tween20	Sigma-Aldrich	9005-64-5	
anti-eIF4E antibody	abcam	ab76256	
anti-Bax antibody	abcam	ab53154	
anti-cytochrome C antibody	abcam	ab90529	
secondary antibody (anti-Rabbit IgG H&	abcam	ab150077	

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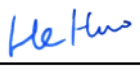
Signature:	
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Dear Editor, Dr. Bing Wu,

Thank you very much for your efforts on handling our manuscript "Photostimulation by femtosecond laser activates extracellular-signal-regulated kinase (ERK) signaling or mitochondrial events in target cells" [JoVE59661]. The valuable comments from editor and reviewers are highly appreciated. We revised the ambiguous statements and figures in the previous version and provided more detailed information for experimental protocols and technical demonstrations. In this response letter, explanations to all the concerns from editor and reviewers are shown as following. I am pleased to submit it along with the revised manuscript to JOVE for your further consideration.

Responses to Editorial comments:

Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Our response:

We have corrected the grammatical mistakes in the revised manuscript.

Comments:

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Our response:

Thanks for reminding the copyright of related figures. According to this comment, we changed the figures to ensure all figures are the initial data for publication.

Comments:

3. The highlighted protocol steps are over the 2.75 page limit (including heading and spacing). Please highlight fewer steps for filming.

5. Please do not highlight not for filming.

6. Step 3.4: Step 2 is referenced here. In order to film this step, step 2 need to be highlighted.

7. Step 7.1: Step 4.1 is referenced here. In order to film this step, step 4.1 need to be highlighted.

8. Step 7.2: Steps 6.3 and 6.4 referenced here. In order to film this step, step 6.3 and 6.4 need to be highlighted.

Our response:

According to those comments, we re-highlighted the protocol steps within 2.75 page limit and other format requirements for filming.

Comments:

4. Please ensure that the references appear as the following:

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Our response:

We revised the reference format accordingly.

Comments:

9. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.

Our response:

According the iThenticateReport, we revised the significant overlap sections in our manuscript.

Thank you for all those kind information.

Responses to Reviewers' comments:

Reviewer #1:

Comments:

-Authors use a Fianium laser at 50 MHz repetition rate. This is quite an unusual source, normally researchers have easier access to Ti:Sapphire oscillators used for multiphoton microscopy (80 MHz). Are these systems adapted for this procedure? What are the minimal laser requirements needed for implementing the stimulation in terms of pulse energy, pulse duration, etc.? These are essential information for interested researchers.

Our response:

Thank you for this comment. This concern might root from our misleading presentations.

In our experiments, both Ti: Sapphire oscillators (MaiTai, SpectraPhysics) and femtosecond fiber lasers are used and can work for the photostimulation. In theory, the photostimulation can effectively achieve the results shown in this manuscript as long as the multiphoton effect is high enough while the side effect (thermal effect and cavity effect) does not harm the cells significantly. In this regard, when the parameters of the femtosecond laser pulses, like wavelength, pulse width, and pulse energy, were changed, the mean power should be tuned accordingly. But the cellular responses would not be quite different. The most significant variation of the cellular responses comes from the multiphoton excitation efficiency that changes along with the optical parameters. For example, the pulse width and wavelength of the fiber laser are both much longer than the Ti: S laser (120 fs vs. 65 fs, 1040 nm vs. 800 nm). Generally at the same mean power, the multiphoton excitation efficiency from the Ti: S laser is much higher than it by the fiber laser (much higher transient peak power and single photon energy). Therefore, the similar cellular responses can be achieved by the Ti: S laser at lower power. The thermal effect is also smaller by it. But at higher laser power, the Ti: S laser can provide much higher multiphoton efficiency to ionize the cell inside the focus that may damage it. The fiber laser in the previous version was only an example. In the revised manuscript, we provide some representative results by using a Ti: Sapphire femtosecond laser (810 nm, 65 fs, 80 MHz), as shown in Figure 5 and 6. We also add the explanations and discussions to show the points above in the manuscript.

In general, according to our previous studies, the cellular molecular activity is mostly induced by multiphoton exaction[1-5]. Hence the pulse width should be short enough to provide a high peak power for high nonlinear exaction efficiency while with little side effect especially heat thermal effect[6]. The typical pulse width for two-photon microscopy, < 200 fs, is an appropriate choice. The repetition rate of the laser is not a key factor since the thermal accumulation at MHz level does not varies a lot. The peak power density of each laser pulse, after focusing, should be around 10^{11}~10^{12} W/cm^2. The laser power and stimulation duration (total incident energy) can provide different cellular molecular responses. It should be noted that the different cellular structures are sensitive to the total incident energy and transient power. The laser power and stimulation duration for ERK activation are quite

different from them that work for eliciting mitochondrial events. We provide more discussions in the revised manuscript.

References:

1. He, Hao, et al. "Manipulation of cellular light from green fluorescent protein by a femtosecond laser." *Nature Photonics* 6.10 (2012): 651.
2. Wang, Yisen, et al. "All-optical regulation of gene expression in targeted cells." Scientific reports 4 (2014): 5346.
3. Wang, Shaoyang, et al. "Photoactivation of Extracellular‐Signal‐Regulated Kinase Signaling in Target Cells by Femtosecond Laser." Laser & Photonics Reviews 12.7 (2018): 1700137.
4. Wang, Yintao, et al. "Photostimulation by femtosecond laser triggers restorable fragmentation in single mitochondrion." Journal of biophotonics 10.2 (2017): 286-293.
5. Shi, Fan, et al. "Mitochondrial swelling and restorable fragmentation stimulated by femtosecond laser." Biomedical optics express 6.11 (2015): 4539-4545.
6. Vogel, Alfred, et al. "Mechanisms of femtosecond laser nanosurgery of cells and tissues." Applied Physics B 81.8 (2005): 1015-1047.

Comments:

-Authors mention often the use of a fast shutter. What fast means in this context? Capable of isolating single pulses at 50 MHz? Or just a mechanical shutter with ms response time?

Our response:

Sorry for our confusing statement. The “fast” is relative to the cellular responses, not to the laser. In our protocol, the stimulation duration is at millisecond level. A mechanical shutter with ms response time is enough to apply. We revised related description in our manuscript.

Comments:

-The alignment of an external laser through a scanning system is a very delicate procedure, the authors provide some useless and redundant instructions (e.g., "turn on laser and microscope") but there is no real suggestions for the alignment procedures (use of reference pinholes, which mirror to steer and in which order, how to ensure the collimation between the two beams, etc.). Neither they discuss the choice of the proper optics for the set-up.

Our response:

Thanks for indicating the ambiguous descriptions in previous manuscript. According to this comment, we presented more detailed information of the establishment of the photostimulation system.

Comments:

Similarly, in the biological procedures for sample preparation, stimulation, and imaging I observe a lack of details related to the specificity of this procedure. How

we define the region for stimulation? What is the signature of laser cell-damage (leading for instance to an increase in autofluorescence) and how can one use this information to adapt the dwell time, pulse energy, etc. for the stimulation? I suggest providing a table comparing the different protocols described with the interval for the corresponding laser specs.

Our response:

To determine the stimulation location in the mitochondrial stimulation experiments, clearly any mitochondrial tubular structure, if selected, is stimulation location. To activate ERK signaling in cells, normally the stimulation region was located in the ER part of the cell, which can be easily found in the cytoplasm close to the nucleus under the bright-field microscopy (the nuclear region of most cell types can be distinguished from cytosol).

To further determine the stimulation area, in Stim-A mode, the photostimulation region is determined by the fixed laser focus, ~ 2 μm in diameter. In Stim-B mode, the stimulation region is pre-designed as any shape but usually a small scanning area (~ 2×2 - 3×3 μm^2). It can be set in the cytosol. But in the ER region close to nucleus can provide higher excitation efficiency due to higher Ca^{2+} depletion effect from ER.

Generally the stimulation efficiency conflicts with cell safety. Higher laser power, longer stimulation duration, larger total incident laser energy, can improve the stimulation efficiency while cells may be damaged. The cell safety can not be determined or described by only one parameter.

The compromise range of laser power should be clearly addressed. However, it should be noted that cell damage and photostimulation effect is not only dependent on laser power but simultaneously influenced by other stimulation parameters like the region, duration, and wavelength. For example, mean power over 60 mW (810 nm) or 120 mW (1040 nm) and exposure duration longer than 1 s can induce irreversible damage to cells by Stim-A mode. But if decreasing the stimulation duration to 0.1 s, the safe power at 810 nm can be as high as 80 mW. The stimulation with an average power higher than 40 mW (810 nm) or 80 mW (1040 nm) and exposure duration over 0.2 s may induce significant damage like irreversible fragmentation in stimulated mitochondrion or even in all mitochondria across the whole cell. In Stim-B mode, except for the mean power and stimulation duration, another factor may affect the photo-activation efficiency and cell damage is the stimulation area. The femtosecond laser stimulation may cause cell damage by delivering on a small stimulation area, but the same stimulation may fail to activate ERK by delivering on a larger stimulation area. Therefore, we can not provide simply safe parameters. But we add tables and more detailed discussions to show some typical examples for reference.

Reviewer #2:

Comments:

Without a reliable control the experiments have not proved which component can be stimulated under 1 W power of 1040 nm wavelength light.

Our response:

Sorry the ambiguous description. At first, the laser output power is 1 W. But the power used for stimulation will be tuned accordingly. We have provided a recommended power level range for stimulation in revised manuscript.

For mitochondrial stimulation, the mitochondrial tubular structure is stimulated by the laser. In Stim-A mode, the laser focus position is fixed in the center of the FOV that can be localized before experiments. To be convenient, the focus position can be labeled in the bright-field microscopy by the CCD camera (as shown in Figure 2) or fluorescent imaging window to aid for moving the target mitochondrial structure on the focus.

For ERK activation, in our protocol, we recommend to deliver the photostimulation on the ER region in Hela cells to achieve a high ERK activation efficiency and little cell damage. The ER region can be easily distinguished from cytoplasm or nucleus under bright-field microscopy by a phase-contrast objective. The photostimulation region is recommended to select the area in ER close to the nucleus. According to previous works[1, 2], the photostimulation in that area can deplete the Ca^{2+} store in ER effectively and activate the CRAC channel. Then the Ca^{2+} influx can activate ERK signaling pathway subsequently.

References:

1. He, Hao, et al. "Manipulation of cellular light from green fluorescent protein by a femtosecond laser." Nature Photonics 6.10 (2012): 651.
2. Wang, Shaoyang, et al. "Photoactivation of Extracellular‐Signal‐Regulated Kinase Signaling in Target Cells by Femtosecond Laser." Laser & Photonics Reviews 12.7 (2018): 1700137.

Comments:

For fluorescence imaging of Hela cell it is used a confocal microscope with the excitation wavelength of 488nm, 532nm and 635nm respectively.

Which is the collection wavelength interval? Which is the focus area and penetration depth of the femtosecond laser?

Our response:

To acquire the green (excited by 488 nm), yellow (excited by 532 nm), and red (excited by 635 nm) fluorescence most effectively, we used dichroic mirrors to split the fluorescence at < 560 nm, 560 - 625 nm and > 625 nm respectively. To acquire an optimal fluorescence blocking of other channels, the wavelength intervals of those filters in the 3 channels are 520 ± 25 nm; 600 ± 25 nm; and 670 ± 40 nm respectively. However, the fluorescent imaging would not be significantly different if the intervals are changed as long as in the green/yellow/red ranges.

The diameter of femtosecond laser focus in cell is around 2 μm (Figure 2). In Stim-B mode, femtosecond laser beam is coupled with the excitation laser beam and works as two-photon microscopy. Hence the focus of femtosecond laser at imaging plane is similar to the traditional multi-photon microscope. In the z-direction (vertical direction), the focus of femtosecond laser can be set in any plane by tuning the distance between two lenses of the relay scope in the vertical direction (the laser propagation direction, vertical to the confocal imaging plane, Figure 1). In our experiment, we adjust the distance between two lenses to ensure the focus of femtosecond laser is on the confocal imaging plane through bright-field imaging of CCD.

Comments:

Which is the dimension/area of the cellular component intended to be stimulated?

Our response:

For mitochondria signal activation, we focus femtosecond laser on anywhere of the selected mitochondrial tubular structure to vibrate mitochondrial physiological status. The diameter of femtosecond laser focus is around 2 μm.

For ERK activation, as discussed above, we intend to deliver photostimulation at ER region in target cells to achieve a high ERK activation efficiency and little cell damage. In Stim-A mode, photostimulation is performed as a single point stimulation. The diameter of femtosecond laser focus is around 2 μm. In Stim-B mode, the stimulation region can be designed as any shape but usually a small scanning area (~ 2×2 - 3×3 μm^2) is used to achieve a high stimulation efficiency and little cell damage.

Comments:

"Photostimulation by femtosecond laser activates extracellular-signal-regulated kinase (ERK) signaling or mitochondrial events in target cells"

...and several other cellular components. It is mentioned that "femtosecond-laser stimulation can directly activate multiple cell processes"

exactly this is the problem in controlling this stimulation. We do not know which process is stimulated.

It is speculative to assign the stimulation to a specific process once the detection of confocal microscopy does not assure a required resolution to follow the biological process.

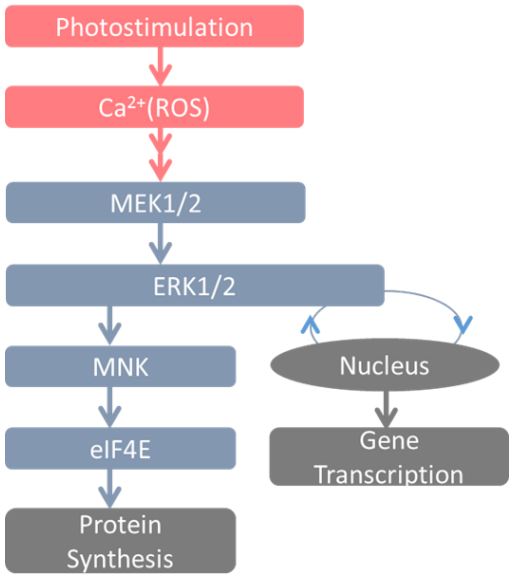
Our response:

The sentence “femtosecond-laser stimulation can directly activate multiple cell processes” provided an ambiguous statement in abstract section. We revised this sentence more clearly and straight-forward.

In introduction section, we briefly explained that femtosecond laser stimulation can induce Ca^{2+} release and a series of cellular molecular signaling events, including NFAT and ERK signaling pathway because they are both the downstream signaling of cell Ca^{2+}. The activation of those signaling are all cellular responses. In theory, they can be activated simultaneously. In this protocol, what we present is a part of cellular responses of laser stimulation. We detailed present the procedures to use

femtosecond laser to affect ERK signaling and mitochondrial signaling events includes mitoflash, mitochondrial morphology changes, mitochondrial membrane potential and Bax or cytochrome C changes. We also briefly discuss that ERK singling pathway can be activated by femtosecond laser stimulation, as the following figure[2].

Photo-activated ERK signaling and mitochondrial signaling events are on the same time and space scale with those processes induced by chemicals[3, 4]. Therefore, the confocal microscopy can prove sufficient resolution to monitor the biological process.



RS Figure 1. A simplified proposed photoactivated ERK pathway demonstration. MEK: mitogen-activated extracellular signal-related kinase. MNK: MAPK-interacting kinases[2].

References:

3. Costa, Mario, et al. "Dynamic regulation of ERK2 nuclear translocation and mobility in living cells." Journal of cell science 119.23 (2006): 4952-4963.
4. Wang, Wang, et al. "Superoxide flashes in single mitochondria." Cell 134.2 (2008): 279-290.

Comments:

Minor Concerns:

1)Although HeLa cell line is well-known, it is required to show the abbreviation when appear first in the text: HeLa cell (cell line derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks; Ref: Scherer, W.F.; Syverton, J.T.; Gey, G.O. (1953). "Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix". Journal of Experimental Medicine. 97 (5): 695-710. doi:10.1084/jem.97.5.695. PMC 2136303. PMID 13052828.)

Our response:

Thanks for reminding this detailed information. We added relevant illustration about Hela cell line in revised manuscript.

Comments:

2)This sentence is confusing: "In current stage, there exist rare methods that can control cellular molecules directly without optogenetics."

Our response:

We revised this ambiguous sentence to make a clear statement.

Comments:

3)Figure 1

It would be useful the specification of which femtosecond laser was used:
wavelength: 1040 nm, repetition rate: 50 MHz, pulse width: 120 fs, maximum output average power: 1 W

4)Figure 2 requires two images of the same area with fs "on" and "off".
In the present figure 2 the indicated by arrow area does not convincingly show the localization of femtosecond laser focus on cell.

5) The figure captions 5 and 6 require the explanation of which excitation wavelength and collection wavelength were used.

Our response:

Thanks for those valuable remarks. We revised the figures and figure legends accordingly.