We describe a protocol for imaging a mitochondrial fluorescence transient increase event (Mitoflash) in live cardiomyocytes using a confocal microscope. Mitoflash, detected by mitochondria-targeted circularly permuted fluorescent protein (mt-cpYFP), can be used to assess mitochondrial respiration function in situ. The protocol is also suitable for live-cell imaging of other adherent cells, including fibroblasts and hepatocytes.
Protocol for Imaging of Mitoflashes in Live Cardiomyocytes

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SUMMARY
We describe a protocol for imaging a mitochondrial fluorescence transient increase event (Mitoflash) in live cardiomyocytes using a confocal microscope. Mitoflash, detected by mitochondria-targeted circularly permuted fluorescent protein (mt-cpYFP), can be used to assess mitochondrial respiration function in situ. The protocol is also suitable for live-cell imaging of other adherent cells, including fibroblasts and hepatocytes.
For complete details on the use and execution of this protocol, please refer to Gong et al. (2014) and Gong et al. (2015).

BEFORE YOU BEGIN
Prepare Solutions and Grease

− Timing: 2–3 h

1. Prepare the necessary solutions according to the Materials and Equipment section.
2. Prepare a high vacuum grease, 20 mL syringe without a needle, a fine-tip forceps, a Pasteur pipette, one quick-release magnetic image chamber, 25 mm sterilized circle microscope coverslips (Figure 1).

Alternatives: The water circulation magnetic chamber (WC-R25, KYODO, Japan) is an alternative chamber. You can also use commercial glass-bottom dishes for cell culture; it is more expensive for large-scale live-cell imaging.

Note: We do not suggest mounting the coverslips on a slide for live-cell imaging, especially for adult cardiomyocytes. The solution dries quickly and will lead to the death of adult cardiomyocytes.

Prepare Cells

− Timing: 1.5–5 h

1. Prepare freshly isolated or cultured cardiomyocytes according to our step-by-step STAR Protocol (Tian et al., 2020. https://star-protocols.cell.com/protocols/103) or other protocols before you start the study.
Note: Cardiomyocytes isolation only needs 1.5–2 h. The isolated cardiomyocytes can be incubated for 2–3 h at 37°C before cultivation.

Pause Point: The cardiomyocytes can wait for up to 2–3 h to culture after isolation.

Note: This protocol is primarily for adult cardiomyocytes. Cardiomyocyte cell lines and other cell types can also be assessed following this protocol. We have validated this protocol on neonatal cardiomyocytes, H9c2 cells, and A549 cells and NSC-34 cells. The cell number, centrifuge speed, and time are variable for different cell types.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, Peptides, and Recombinant Proteins | | |
| M199 | Sigma-Aldrich | Cat# M2520 |
| Pen/Strep (100X) | Thermo Fisher Scientific | Cat# 10378016 |
| Insulin-transferrin-selenium-X | Thermo Fisher Scientific | Cat# 51500056 |
| Fetal bovine serum | Thermo Fisher Scientific | Cat# 12483020 |
| NaHCO₃ | Sigma-Aldrich | Cat# V900182 |
| Glutathione | Sigma-Aldrich | Cat# G6013 |
| Creatine | Sigma-Aldrich | Cat# C3630 |
| L-carnitine | Sigma-Aldrich | Cat# C0158 |
| Taurine | Sigma-Aldrich | Cat# T8691 |
| NaCl | Sigma-Aldrich | Cat# V900058 |
| KCl | Sigma-Aldrich | Cat# V900068 |
| CaCl₂ | Sigma-Aldrich | Cat# V900266 |

(Continued on next page)
MATERIALS AND EQUIPMENT

**Alternatives:** Mito-Hyper Addgene (Cat# 136470) and mt-cpYFP (Addgene Cat# 51868) can be used instead of the Ad-mt-cpYFP mentioned here.

**Alternatives:** Most confocal microscopes can image mitoflashes, such as Leica TCS SP8.

**Note:** Prepare all solutions using 18.2 Ω MilliQ sterilized H2O.

- **1 M NaCl:** Dissolve 29.22 g NaCl in 500 mL ddH2O. Store at room temperature (23°C–26°C).
- **0.5 M KCl:** Dissolve 0.7475 g KCl in 20 mL ddH2O. Store at room temperature (23°C–26°C).
- **100 mM CaCl2:** Dissolve 0.5549 g CaCl2 in 50 mL ddH2O. Store at room temperature (23°C–26°C).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| D-Glucose           | Sigma-Aldrich | Cat# G8270 |
| MgSO4·7H2O          | Sigma-Aldrich | Cat# V900270 |
| KH2PO4              | Sigma-Aldrich | Cat# V900041 |
| HEPES               | Sigma-Aldrich | Cat# V900477 |
| Pyruvate            | Sigma-Aldrich | Cat# P2256 |
| Ad-mt-cpYFP         | This study | N/A |
| Ethanol Absolute    | Sigma-Aldrich | Cat# 51976 |

**Experimental Models: Organisms/Strains**

- **SD rat** Shanghai SLAC | Cat# SlacSD

**Software and Algorithms**

- **ZEN** Zeiss | [https://www.zeiss.com/microscopy/int/software-cameras.html](https://www.zeiss.com/microscopy/int/software-cameras.html)
- **ZEN core** Zeiss | [https://www.zeiss.com/microscopy/us/products/microscope-software.html](https://www.zeiss.com/microscopy/us/products/microscope-software.html)
- **Image J (Rasband, NIH)** NIH | [https://imagej.nih.gov/ij/download.html](https://imagej.nih.gov/ij/download.html)

**Other**

- **High vacuum grease** DOW CORNING | Cat# HVG
- **20 mL Syringe** Huanxi Medical | Cat# 66949
- **Fine-tip forceps** Sangon Biotech | Cat# FS19021
- **Pasteur pipette** NEST | Cat# 318314
- **15 mL Centrifuge tube** Thermo Fisher Scientific | Cat# 339651
- **6 well plate** Thermo Fisher Scientific | Cat# 140657
- **Quick-Release Magnetic Chambers** Warner Instruments | Cat# 64-1947
- **Microscope coverslip** MARIENFELD | Cat# AP-1111650
- **Bottle Top Filter** ThermoFisher | Cat# 2903345
- **Hemocytometer** MARIENFELD | Cat# AP-0650010
- **Water bath** YIHENG China | Cat# HWS-12/24
- **Inverted microscope** Leica | DMi8
- **ZIELS LSM 880 Confocal Laser Scanning Microscope** ZEISS | ZEISS LSM 880
- **pH meter** Mettler Toledo | FE20 plus
- **Kimwipes** Kimberly-Clark Professional | Cat# 06-666A
- 0.25 mM KH₂PO₄: Dissolve 0.34 mg KH₂PO₄ in 10 mL ddH₂O. Store at room temperature (23°C–26°C). Dilute to 0.25 mM with ddH₂O before use.
- 100 mM MgSO₄·7H₂O: Dissolve 2.4647 g MgSO₄·7H₂O in 100 mL ddH₂O. Store at room temperature (23°C–26°C).
- 100 μM TMRM solution: Dilute the 10 mM TMRM stock solution with DMSO to 100 μM work concentration and store at −20°C for up to 6 months.
- 500 mM Pyruvate: Dissolve 550.2 mg pyruvate in 10 mL ddH₂O. Neutralize with 5 M KOH and check pH. Dispense into 100 μL aliquots, store at −20°C for up to 3 months.
- 40 μg/ml Laminin preparation: Thaw laminin at 2°C–8°C, dilute the 1 mg laminin into 40 μg/mL with 25 mL sterilized cold DPBS (stored at 4°C), dispense into several working aliquots (100 μL, 500 μL, 1 mL) and store at −20°C for up to 6 months.

△ CRITICAL: Laminin needs to thaw slowly at 2°C–8°C. If the product was thawed at room temperature (23°C–26°C), it is easy to form gels; it cannot be reactivated for use.

### M 199 Medium

| Reagent          | Final Concentration | Amount   |
|------------------|---------------------|----------|
| M 199            | n/a                 | 1 bag (9.5 g) |
| NaHCO₃           | ~2.2 g/L            | ~2.2 g   |
| Glutathione      | 10 mM               | 3.073 g  |
| BSA              | 0.2 g/L             | 0.2 g    |
| ddH₂O            | n/a                 | ~1,000 mL|
| Total            | n/a                 | 1,000 mL |

**Note:** Adjust the pH to 7.4 with NaHCO₃, filter with a 0.22 μm bottle top filter.

### Culture Medium

| Reagent                          | Final Concentration | Amount/Volume |
|----------------------------------|---------------------|---------------|
| M 199 medium                     | n/a                 | 93.89 mL      |
| Pen/Strep (100×)                 | 1×                   | 1 mL          |
| Creatine                         | 5 mM                | 74.58 mg      |
| L-carnitine                      | 2 mM                | 32.24 mg      |
| Taurine                          | 5 mM                | 62.58 mg      |
| Insulin-transferrin-selenium-X (1003) | 0.1%              | 0.1 mL        |
| Blebbistatin (100 mM)            | 10 μM               | 10 μL         |
| Total                            | n/a                 | 100 mL        |

**Note:** Warm the medium to room temperature (23°C–26°C) before use.

### Krebs-Henseleit Buffer (KHB)

| Reagent       | Final Concentration | Amount/Volume |
|---------------|---------------------|---------------|
| NaCl (1 M)    | 138.2 mM            | 138.2 mL      |
| KCl (0.5 mM)  | 0.0037 mM           | 7.4 mL        |
| CaCl₂ (100 mM)| 0.25 mM             | 2.5 mL        |
| KH₂PO₄ (0.25 mM) | 0.0012 mM | 4.8 mL        |
(Continued on next page)
### Coat Coverslips

**Timing:** 1–1.5 h

Adult cardiomyocytes have poor adhesion, coating coverslips with laminin can enhance adult cardiomyocytes adhesion.

1. Place a diameter 25 mm circular coverslip to a 6 well plate or 35 mm dishes by a fine-tip forceps (Figure 2A).

   **Note:** Compared with 35 mm glass-bottom dishes, coverslip plus plate is much cheaper. Coverslips need to be autoclaved in advance.

2. Coat the coverslips with 100 μL 40 μg/mL laminin, carefully spread the laminin to evenly over the coverslip using the pipette tip (Figures 2B and 2C).

   **Alternatives:** Add 500 μL laminin to the coverslips, moving plates backward and forward, then right to left to right, repeat 2–3 times, let laminin spread evenly over the coverslip. It saves you time, but the cost is higher than add 100 laminins.

   **Note:** Plate coating should be done during cardiomyocyte collection (Lundgren et al., 1988). This step is not necessary for cell types that have strong adhesion.

3. Gently put the plate with coated coverslip into a 37°C incubator for at least 1 h, but no more than 3 h.

### Culture Cardiomyocytes

**Timing:** 2.5–3 h

Cardiomyocytes cultivation is for infecting Adeno-mt-cpYFP.

4. Transfer freshly isolated cardiomyocytes according to our step-by-step STAR protocol (Tian X et al., 2020. [https://star-protocols.cell.com/protocols/103](https://star-protocols.cell.com/protocols/103)) or other cultured cells to a 15 mL conical tube, centrifuge at 30 × g, 30 s.

   **Note:** The speed and time of centrifuge vary from different cell types. Cardiomyocytes are far larger than cell lines, 30 s is enough to get the cell sediment.
5. Discard the supernatant using a pipette and resuspend the pellet in 4–8 mL pre-warmed M199 culture medium.

6. Count cardiomyocytes using a traditional Hemocytometer and dilute into $2.5 \times 10^5$ mL with M199 culture medium.

7. Take out the coated plate and discard laminin with a pipette.

**CRITICAL:** Do not let the coverslip dry. The dry laminin cannot be reactivated for cell attachment.

8. Add 200 $\mu$L cardiomyocyte suspension at a density of $2.5 \times 10^5$ cells to a contained coverslip center, and the cells will automatically spread on the coverslip (Figure 3A).

9. Gently put the plate into a 37°C, 5% CO₂ incubator, wait for at least 2 h, let cardiomyocytes attached to the coverslip.

**CRITICAL:** Appropriate cell number is very important for live-cell imaging. Too many will lead to a hard observation of a single cell after proliferation under the confocal microscope.

Pause Point: The plated cardiomyocytes can wait for up to 4 h to infect the virus.

**Infect Adeno-mt-cpYFP**

**Timing:** 2 days

Express mitoflash biomarker, mt-cpYFP, in the mitochondrial matrix of cardiomyocytes.

10. After 2–4 h incubation, take out an aliquoted Ad-mt-cpYFP virus from −80°C and thaw it on ice.

**Note:** The concentration of Ad-mt-cpYFP must be determined, aliquoted to 5–10 $\mu$L, and stored at −80°C to avoid repeated freezing and thawing, which will result in a decreased activity. If there are residual, return it to −80°C quickly.

**Note:** It has been reported that Mito-Hyper can detect the same event as mt-cpYFP (Quatresous et al., 2012). It may as a viable alternative to cpYFP after extensively verified. We only used mt-cpYFP to detect mitoflash so far.

11. Gently discard the 200 $\mu$L medium and add 1 mL fresh M199 culture medium (Figure 3B), the unattached cells will be discarded.
12. Add proper Ad- mt-cpYFP (50 MOI) into the medium, gently move plates backward and forward, then right to left to right, repeat 2–3 times, let the virus spread evenly, incubate at 37°C, 5% CO₂ for 1–2 h.

**Note:** Add the virus in 1 mL medium can enhance the concentration of the virus to improve the infection efficiency of virus. Generally, the MOI of recombinant adenovirus from 20 to 100 is enough to infect cells (Gong et al., 2014).

13. Take out the plate and add 1 mL fresh M199 culture medium to each well (total 2 mL), incubate at 37°C, 5% CO₂ for 48 h, let cpYFP expressing.

**Note:** The virus can be left in the medium. It is not necessary to replace the medium the next day. Adult cardiomyocytes can be cultured at 37°C, 5% CO₂ for up to 5–6 days with the medium change every 2 days (Mitcheson et al., 1998; Liu et al., 2019).

### Pause Point
Mitoflashes imaging of cardiomyocytes can wait for up to 62 h after infection.

### Confocal Imaging

**Timing:** 1–1.5 h

Monitor and take 2-dimension images of mitoflashes in adult cardiomyocytes.

14. Pre-warm the KHB buffer to room temperature (23°C–26°C).
15. Thaw TMRM work solution and add 2 μL per well (1:1,000) to stain mitochondrial membrane potential in a 37°C incubator for 20–30 min.

**Note:** The TMRM not only serves to identify mitoflashes but also helps to identify the localization of cpYFP.

16. Set the imaging parameters of Zen software: Dual excitation images of mt-cpYFP were taken by sequential excitation at 488 nm and 543 nm, and emission was collected at 505–545 nm and >560 nm, respectively. Time-lapse x,y images were acquired at 1,024 × 1,024 resolution for 100 frames and at a sampling rate of 1 s per frame (Figure 4).

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**Figure 3. Adult Cardiomyocytes Culture and Infection**

(A) Add 200 μL cardiomyocytes suspension onto the coated coverslip.
(B) Add 1 mL M199 culture medium.
Note: Scan Mode: frame; Speed: 9; Averaging: number, 1; Gain 450–600; Pinhole 30–200; laser <10%. You’d better start Zen software first, completely turn on the 488 nm laser, 543 nm laser need 3–5 min.

17. Take off the magnetic top of the Quick-Release Magnetic chamber, apply the high vacuum grease around the inside pedestal evenly of the Magnetic bottom (Figure 5A).

Note: The grease must be evenly spread. Otherwise, the solution will leak during the final imaging, which may damage the objective.

18. Using fine-tip forceps to get the coverslip out, put it on a Kimwipes cleaning paper to dry the bottom (without cells face) of the coverslip.

19. Place the coverslip into the magnetic bottom of the imaging chamber and put the magnetic top back (Figures 5B and 5C). The coverslip will be automatically adhered to the chamber by the magnetic power.

△ CRITICAL: To maintain the integrity of the coverslip, hold the edge of the coverslip with forceps. Do not use the fine-tip forceps to press the coverslip, as these can break the coverslips.
20. Add 1 mL KHB buffer with a pipette into the chamber (Figure 5C).

**Note:** Put the imaging chamber on a Kimwipe to check for leakage.

△ CRITICAL: Steps 18–20 must be performed quickly (20–40 s), and carefully, otherwise, adult cardiomyocytes will eventually shrink and die after leaving the solution too long.

21. Add one drop of oil on top of the 63× objective, place chamber on microscope stage above objective (roughly in the center of the field of view).

22. Find and focus the cell sample in the eyepieces, and move it to the center field of vision.

23. Choose only 488 channel to pre-setup relevant imaging parameters (Gain 450–600; Pinhole 30–200; laser <10%) by the live scan.

24. Click on “Live” on the top left corner, then adjust the focal position by rotating the knob until the mitochondria are focused.

25. Adjust laser power, pinhole, gain, digital offset.

**Note:** Scan Mode: frame; Speed: 9; Averaging: number, 1; Gain 450–600; Pinhole 30–200; laser <10%. The laser too strong will damage the cell during a long time two dimensions (2D) scanning. You can use a higher laser for 1D imaging.

26. Choose a rod cardiomyocyte with a clear mitochondrial pattern (Figure 6A) that satisfied you by crop, acquire, and save a 2D image with 100 frames.

**Analyze Mitoflash**

.randn; Timing: 5–30 min

Obtain the frequency and kinetic parameters of mitoflashes.

27. The number of mitoflash was count by eye while playing the 2D images using the Zeiss LSM viewer software (Split xy → Zoom → Slice → repeatedly drag the Play progress bar until you finish the counting).

**Alternatives:** Mitoflashes can be identified with the help of Flashsniper (Li et al., 2012).

28. Videos (Video S1) were generated using the Zeiss LSM viewer software according to 16 frames/s (Export → Series → 16 frames/s → Avi format).
The traces of mitoflash and TMRM (Figures 6B and 6C) of selected mitochondria were generated using Image J (Drag the Play progress bar to identify mitoflashes → Selections tool define the mitochondrial area with mitoflash Plugins → Stack-T-function → Intensity v Time Monitor).

EXPECTED OUTCOMES

Mitochondrial flashes (mitoflashes) are mitochondrial matrix cpYFP fluorescence transient increase events in many cell types. cpYFP act as a novel biosensor of superoxide, which can indicate the primal ROS generated by the mitochondrial electron transport chain. Mitoflash occurring, as a special individual mitochondrial superoxide burst event sensed by cpYFP, is based on the mitochondrial basal ROS increase. Erupting superoxide will activate the mitochondrial permeability transition pore (mPTP) to open, resulting in a depolarization of mitochondrial membrane potential ($\Delta \Psi_m$). The signal of mitoflash includes a modest matrix alkalization. Thus, It can reflect the trend of ROS change but can not accurately quantify the ROS.

Mitoflash is a potent mitochondrial biological marker to monitor mitochondria relevant biological events. It amplifies the signal of respiration active mitochondria and thus made the mitochondria was easily identified from the other static mitochondria (Figure 6B and Video S1). Mitoflashes are always companied by the mitochondrial membrane potential ($\Delta \Psi_m$) decrease due to the mPTP opening (Figures 6B and 6C). The recover time of $\Delta \Psi_m$ is variable, range from 10 to 60 s. Sometimes complete $\Delta \Psi_m$ depolarization...
The frequency of mitoflash has important physiological and pathophysiological significances. Its frequency is closely associated with muscle contraction, cell differentiation, neuron development, and degeneration, lifespan prediction, and wound healing (Wang et al., 2016). The number of typically mitoflashes is varied from different cells. For example, the mitoflash number is $3.8 \pm 0.5$ in adult cardiomyocytes, and $31 \pm 4$ in primary cultured hippocampal neurons (Wang et al., 2008). For the confidence in mitoflash data, we generally analyze at least 18–24 cells from three independent experiments.

Starvation and bioenergetics can remarkably affect the frequency (Figure 7A). The kinetics of mitoflash, including amplitude ($\Delta F/F_0$, represents the level of a superoxide burst), $T_{pk}$ (represents the speed of a superoxide burst), and $T_{50}$ (represents the duration of a superoxide burst) (Figures 7B–7D). These parameters are the kinetics. Any treatments may potentially affect those parameters. We majorly focused on the frequency. Compared with fixed cells, live-cell imaging is necessary for some experimental analysis. At the same time, it is valuable to monitor mitochondrial function in real-time.

LIMITATIONS
This protocol has been designed for imaging in live cells. If you are planning to perfuse live cells with different solutions or to use electrical stimulation of the cells, a commercial imaging chamber such as Warner RC-47FSLP Quick change chamber is required.
TROUBLESHOOTING

Problem 1
The coverslip was broken while putting it into the imaging chamber.

Potential Solution
Be more careful to pick up the coverslip from the plate. Prepare double cell samples on the coverslips. You can use the empty glass to practice a few more times before using cell samples.

Problem 2
Solution leakage

Potential Solutions
Take off the magnetic top, and use the Pasteur pipette to press the edge of coverslip, let the coverslip and chamber adhered tightly (Figure 8). Carefully remove the from the chamber, clean the grease on the chamber, and put enough vacuum grease on the pedestal evenly again. Change the O ring of the magnetic top.

Problem 3
The intensity of the fluorescence suddenly increased.

Potential Solutions
A leakage of KHB buffer may happen, you should remove the chamber from the sample stage, check for leakage, press the coverslip again, and add KHB buffer.

Problem 4
The signal of cpYFP is too weak

Potential Solutions
In our protocol, we add 50 MOI of Ad-mt-cpYFP to infect cells. If the signal of cpYFP is still weak, you can prolong infection time to 62 h or add 100 MOI adenovirus to infect cells. You can also increase the laser or the gain to enhance the signal of cpYFP. Please re-titer the virus if it still does not work.

Problem 5
The signal of cpYFP is too strong
Potential Solutions
Generally, the high fluorescent signal of the construct can be solved by decreasing the laser or the gain. You can also reduce the MOI during virus infection.

Problem 6
Lack of mitoflashes

Potential Solutions
The low viability of cells after culturing may lead to a lack of mitoflashes.

Re-isolate and culture cardiomyocytes.

RESOURCE AVAILABILITY
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guohua Gong (guohgong@tongji.edu.cn).

Materials Availability
Ad-mt-cpYFP used in this study is available from the Lead Contact with a completed Materials Transfer Agreement. Plasmids can be purchased through Addgene (details listed in the Key Resources Table).

Data and Code Availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100101.

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AUTHOR CONTRIBUTIONS
G.-G.H. conceived, designed, and supervised the project. L.-A.Q., Q.-Y., and J.-W.T. conducted most of the experiments and performed data analysis. T.-X.G., and L.-B.L. isolated the cardiomyocytes. Q.-Y., and G.-M. provided valuable suggestions. G.G. and L.-A.Q. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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