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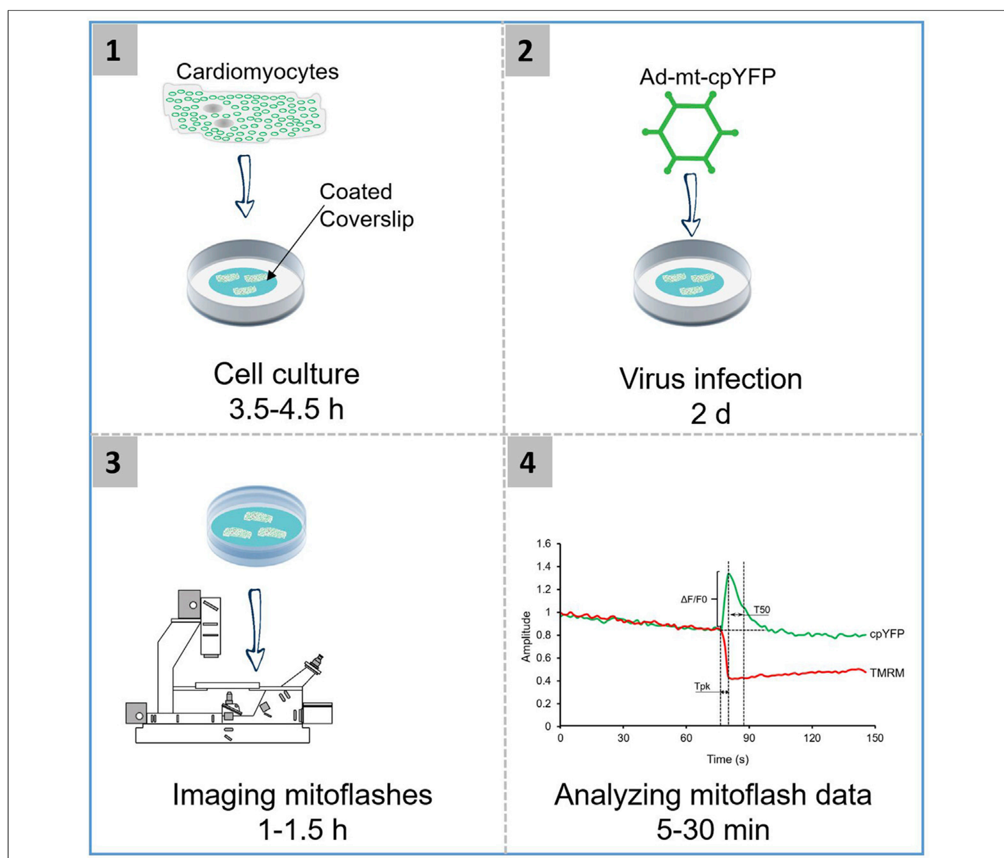
Protocol for Imaging of Mitoflashes in Live Cardiomyocytes



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.

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HIGHLIGHTS

Mitoflash is a superoxide burst event of individual mitochondria

Mitoflash can be detected by a mitochondria target fluorescent protein (mt-cpYFP)

We describe mitoflash imaging in live cardiomyocytes with confocal microscopy.

This protocol can be adapted to other adherent cell types.

Li et al., STAR Protocols *1*, 100101
September 18, 2020 © 2020 The Author(s).
https://doi.org/10.1016/j.xpro.2020.100101

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Protocol for Imaging of Mitoflashes in Live Cardiomyocytes

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https://doi.org/10.1016/j.xpro.2020.100101

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.

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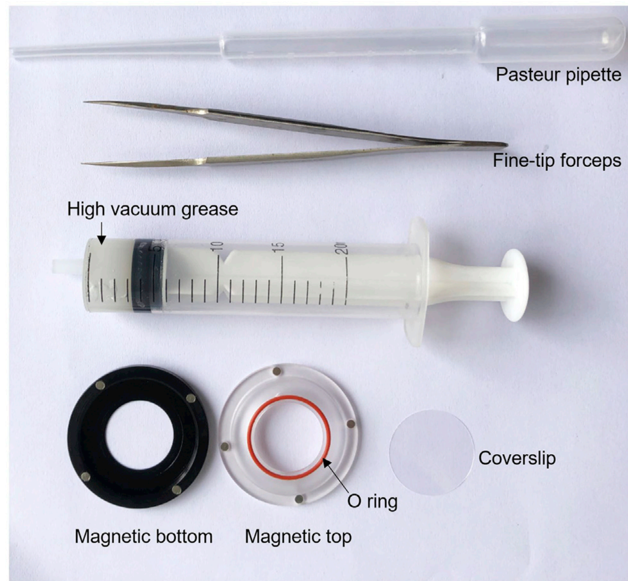

Figure 1. Tools for Live-Cell Imaging

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 (100×)	Thermo Fisher Scientific	Cat# 10378016
Insulin-transferrin-selenium-X	Thermo Fisher Scientific	Cat# 51500056
Fetal bovine serum	Thermo Fisher Scientific	Cat# 12483020
$NaHCO_3$	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
$CaCl_2$	Sigma-Aldrich	Cat# V900266

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D-Glucose	Sigma-Aldrich	Cat# G8270
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# V900270
KH_2PO_4	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
ZEN core	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software.html
Image J (Rasband, NIH)	NIH	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# F519021
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-0111650
Bottle Top Filter	ThermoFisher	Cat# 2903345
Hemocytometer	MARIENFELD	Cat# AP-0650010
Water bath	YIHENG China	Cat# HWS-12/24
Inverted microscope	Leica	DMi8
ZEISS LSM 880 Confocal Laser Scanning Microscope	ZEISS	ZEISS LSM 880
pH meter	Mettler Toledo	FE20 plus
Kimwipes	Kimberly-Clark Professional	Cat# 06-666A

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 H_2O.

- **1 M NaCl**: Dissolve 29.22 g NaCl in 500 mL ddH_2O. Store at room temperature (23°C–26°C).
- **0.5 M KCl**: Dissolve 0.7475 g KCl in 20 mL ddH_2O. Store at room temperature (23°C–26°C).
- **100 mM $CaCl_2$**: Dissolve 0.5549 g $CaCl_2$ in 50 mL ddH_2O. Store at room temperature (23°C–26°C).

- **0.25 mM KH_2PO_4**: Dissolve 0.34 mg KH_2PO_4 in 10 mL ddH_2O. Store at room temperature (23°C–26°C). Dilute to 0.25 mM with ddH_2O before use.
- **100 mM $MgSO_4 \cdot 7H_2O$**: Dissolve 2.4647 g $MgSO_4 \cdot 7H_2O$ in 100 mL ddH_2O. 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 ddH_2O. 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)
$NaHCO_3$	~ 2.2 g/L	~ 2.2 g
Glutathione	10 mM	3.073 g
BSA	0.2 g/L	0.2 g
ddH_2O	n/a	~1,000 mL
Total	n/a	1,000 mL

Note: Adjust the pH to 7.4 with $NaHCO_3$, 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
$CaCl_2$ (100 mM)	0.25 mM	2.5 mL
KH_2PO_4 (0.25 mM)	0.0012 mM	4.8 mL

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Reagent	Final Concentration	Amount/Volume
$MgSO_4 \cdot 7H_2O$ (100 mM)	1.2 mM	12 mL
Glucose	15 mM	2.7024 g
HEPES	21.85 mM	5.2058 g
ddH_2O	n/a	~1,000 mL
Total	**n/a**	**1,000 mL**

Note: Adjust the pH to 7.4 with 5 M KOH, filter with a 0.45 μm bottle top filter, dispense into 50 mL aliquots and store at −20°C.

STEP-BY-STEP METHOD DETAILS

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) 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.

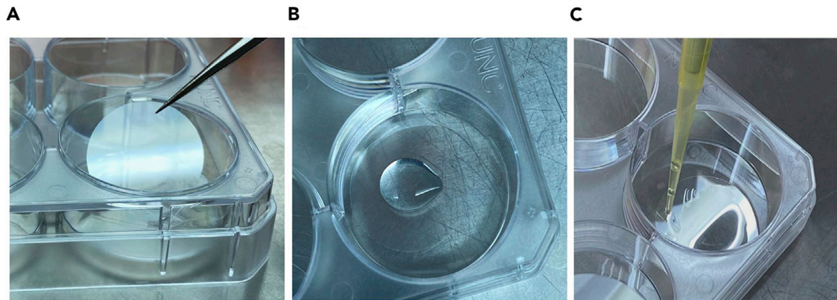

Figure 2. Coating Coverslip with Laminin
(A) Put a coverslip into well.
(B) Add laminin onto coverslip.
(C) Spread the laminin over the coverslip.

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 2.5×10^5/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 μL cardiomyocyte suspension at a density of 2.5×10^5 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% CO_2 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 μL, 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 μL medium and add 1 mL fresh M199 culture medium (Figure 3B), the unattached cells will be discarded.

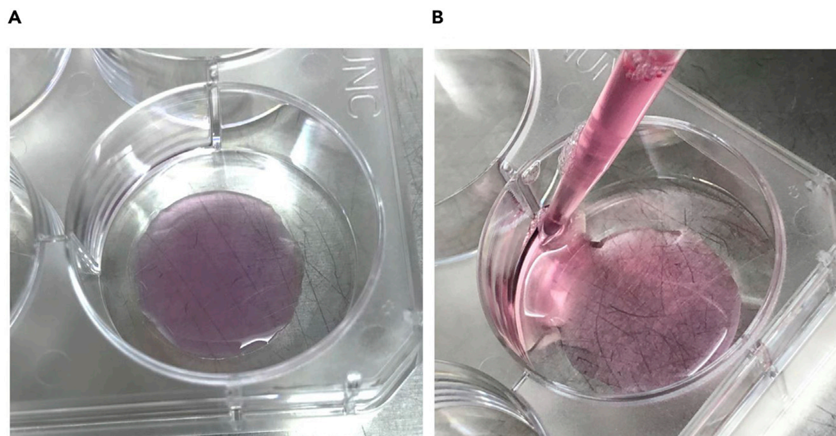

Figure 3. Adult Cardiomyocytes Culture and Infection
(A) Add 200 μL cardiomyocytes suspension onto the coated coverslip.
(B) Add 1 mL M199 culture medium.

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% CO_2 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% CO_2 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% CO_2 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).

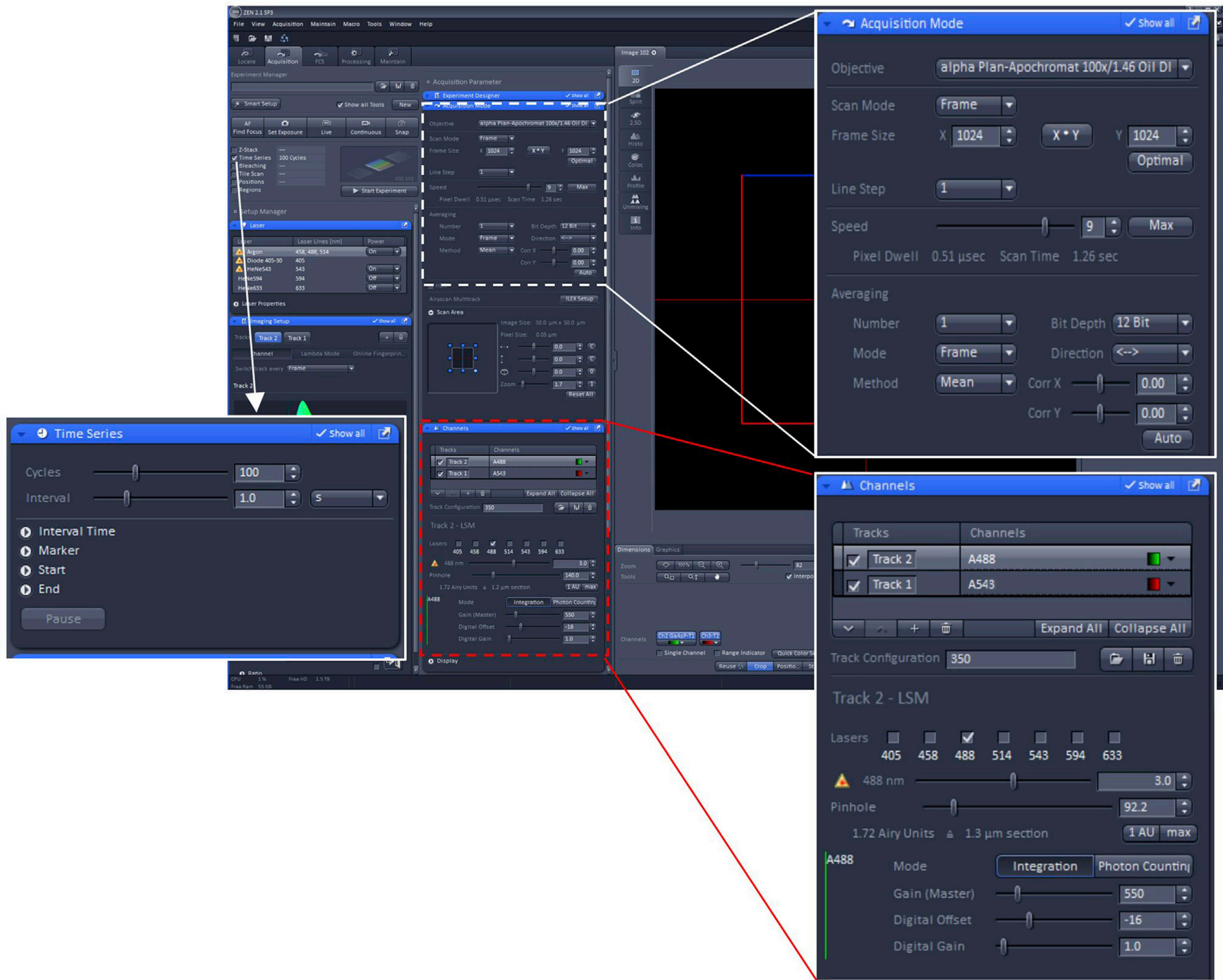

Figure 4. The Imaging Parameters of Zen Software for Imaging Mitoflashes

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.

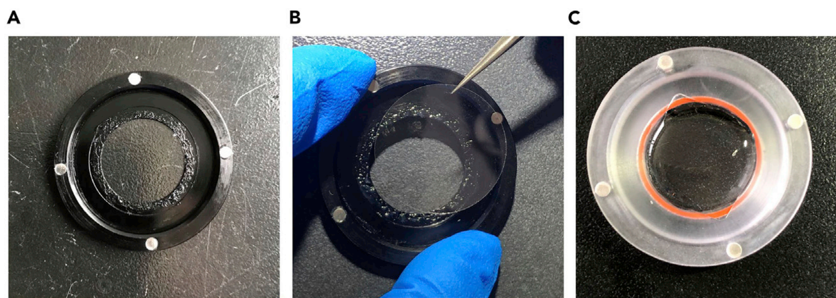

Figure 5. Assemble Coverslip and Magnetic Chamber
(A) The magnetic bottom with the high vacuum grease.
(B) Place the coverslip into the magnetic bottom.
(C) Put the magnetic top back and add 1ml KHB buffer.

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

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).

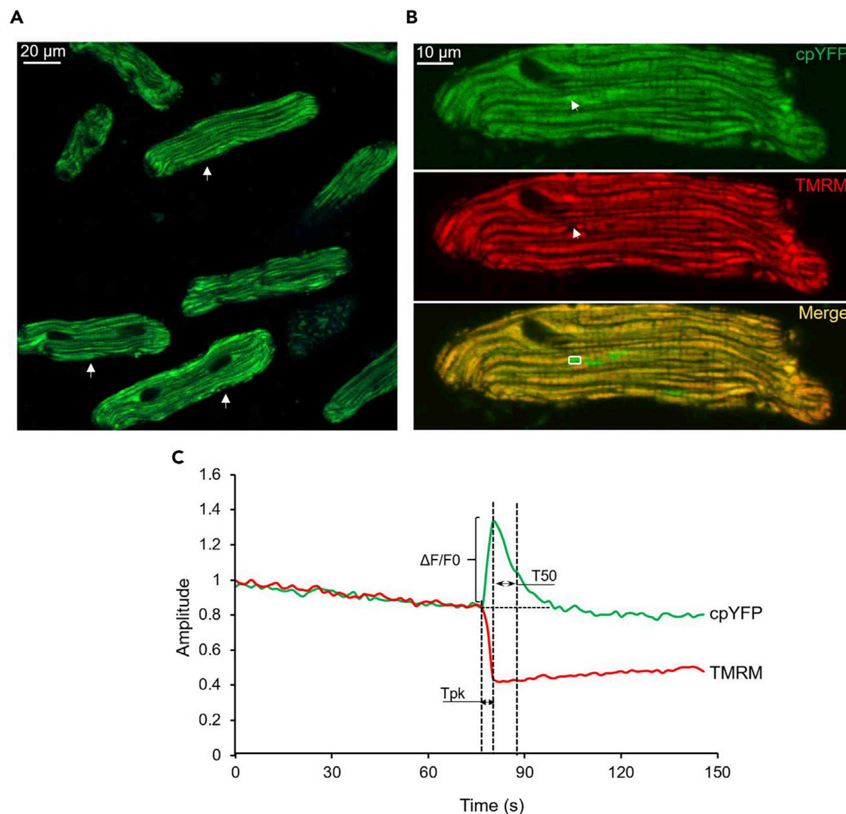

Figure 6. Mitoflashes Analysisin Live Adult Cardiomyocyte

(A) Representative images of adult rat cardiomyocytes expressing cpYFP. The rod cells with clear mitochondrial pattern (marked with white arrow) were used for mitoflash analysis.

(B) Representative images of an adult cardiomyocyte that is expressing cpYFP and stained with TMRM. The chosen mitoflash was marked with white arrow and box.

(C) The traces of cpYFP and TMRM fluorescence. The amplitude ($\Delta F/F_0$), time to peak (T_{pk}) and time of 50% decay (T_{50}) of mitoflash were annotated on the trace.

29. 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 *(ΔΨm)*. 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 (ΔΨm) decrease due to the mPTP opening (Figures 6B and 6C). The recover time of ΔΨm is variable, range from 10 to 60 s. Sometimes complete ΔΨm depolarization

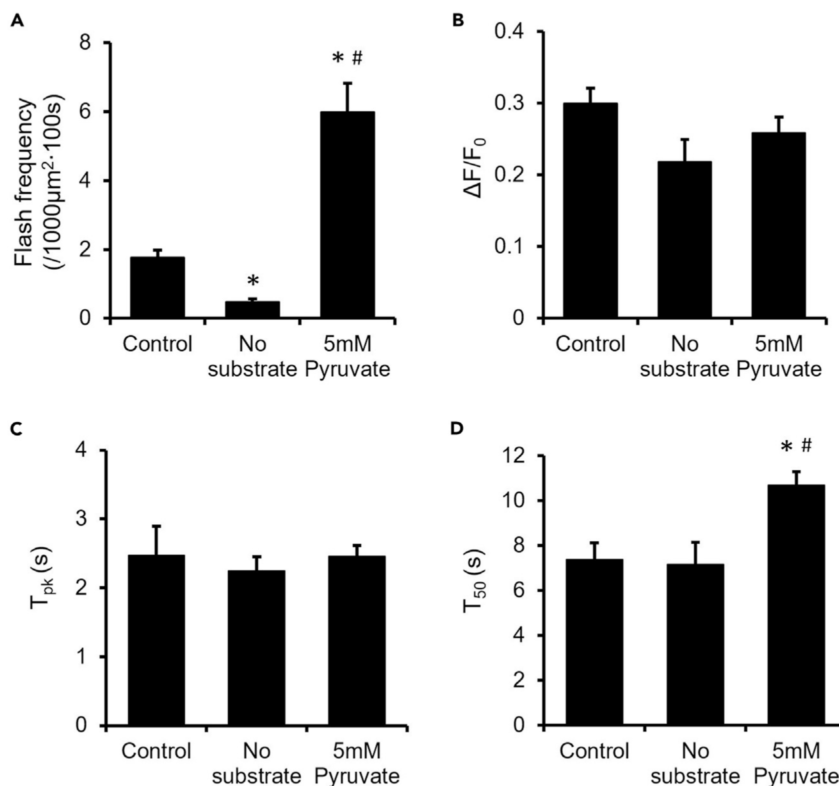

Figure 7. The Substrate Supports Mitoflash Generation in Adult Rat Cardiomyocytes

(A) Mitoflash frequency in normal KHB solution (Control), in solutions without any substrate (No substrate) or with 5 mM pyruvate. Data are mean ± SEM, n= 18–24 cells from three rats. *p <0.01 versus Control, #p <0.05 versus No substrate.

(B–D) Mitoflashes quantified in (A) were further analyzed for amplitude ($\Delta F/F_0$), time to peak (T_{pk}), and time of 50% decay (T_{50}). (B) Summarized data of ($\Delta F/F_0$). (C) Data of T_{pk}. (D) Data of T_{50}. Data are mean ± SEM, n=27–98 flashes from18–24 myocytes. *p <0.01 versus Control, #p <0.05 versus No substrate.

that outlasted the cpYFP signal (Feng et al., 2019). 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 ± 0.5 in adult cardiomyocytes, and 31 ± 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 (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 (Please refer to the calcium transient, Liu et al., 2019) of mitoflashes. 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.

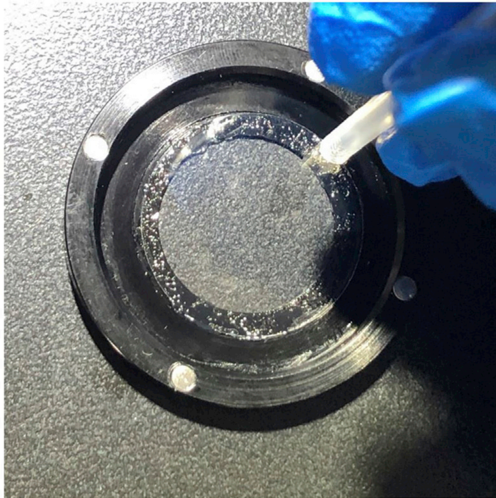

Figure 8. Reassemble Chambers against Leakage

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.

ACKNOWLEDGMENTS

This work was supported partly by the National Key Research and Development Program of China (no. 2018YFA0107102 to G.-G.H.), the National Natural Science Foundation of China (no. 81970333, 31771524 to G.-G.H. and no. 31901044 to Q.-Y.), and the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (no. TP2017036 to G.-G.H.).

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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