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Supplemental Information

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Xianhua Wang, Xing Zhang, Zhanglong Huang, Di Wu, Beibei Liu, Rufeng Zhang, Rongkang Yin, Tingting Hou, Chongshu Jian, Jiejia Xu, Yan Zhao, Yanru Wang, Feng Gao, and Heping Cheng

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XH Wang[1*,†], X Zhang[2*], ZL Huang[1*], D Wu[1], BB Liu[1], RF Zhang[1], RK Yin[1], TT Hou[1], CS Jian[1], JJ Xu[1], Y Zhao[1], YR Wang[1], F Gao, HP Cheng[2†]

[1]State Key Laboratory of Membrane Biology, Peking-Tsinghua Center for Life Sciences, Institute of Molecular Medicine, Peking University, Beijing 100871, China

[2]Department of Aerospace Medicine, The Fourth Military Medical University, Xi'an 710032, China

[*]co-first authors

[†]correspondence authors

Materials and Methods

Reagents

Nigericin, monensin, 2-nitrobenzaldehyde (NBA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 2, 7-dichlorodihydrofluorescein diacetate (DCF) were from Sigma. Tetramethylrhodamine methyl ester (TMRM), Mg^{2+}-Green AM, Rhod-2 AM, mitoSOX, and SNARF-1 AM were from Invitrogen.

Animal care

All procedures were carried out according to the rules of the American Association for the Accreditation of Laboratory Animal Care International and approved by the Animal Care Committee of Peking University (accredited by AAALAC International). This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Adult cardiomyocyte isolation, culture, and adenovirus infection

Single ventricular myocytes were enzymatically isolated from the hearts of adult male Sprague–Dawley rats (200–250 g), as described previously (1). Freshly-isolated cardiomyocytes were plated on culture dishes coated with laminin (Sigma) for 1 h and then the attached cells were cultured in M199 medium (Invitrogen) along with (in mM) 5 creatine, 2 L-carnitine, 5 taurine, and 25 HEPES (all from Sigma). Cells were then infected with adenovirus carrying mt-cpYFP, mt-EYFP, mt-grx1-roGFP2, or mt-pHTomato at m.o.i. of 20 and experiments were performed after 48 – 72 h in culture. Cells of rod - shaped morphology with good indicator expression were chosen for mitoflash experiments.

Cardiomyocyte permeabilization

Adult cardiomyocytes were incubated in Ca^{2+}-free Tyrode's solution consisting of (in mM) 137 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 5.6 glucose, and 20 HEPES (pH 7.35, adjusted with NaOH) for 3 min and then in the internal solution containing (in mM) 100 potassium aspartate, 20 KCl, 0.8 $MgCl_2$, 3 Mg-ATP, 0.5 EGTA, 10 phosphocreatine, 5 U/ml creatine phosphokinase, 10 glutathione, 20 HEPES, 8% dextran (MW 35,000-45,000), and 50

μg/ml saponin (pH 7.2) for 30 sec (2). After permeabilization, cells were incubated in a solution containing (in mM) 100 potassium aspartate, 1.0 $MgCl_2$, 20 KCl, 0.5 EGTA, 10 glutathione, 20 HEPES, 8% dextran (MW 35,000-45,000), 10 KH_2PO_4, 5 succinate, and 0.02 ADP (pH 7.2).

Confocal imaging of mitoflashes

An inverted confocal microscope (Zeiss LSM 710) with a 40×, 1.3 NA oil-immersion objective was used for imaging. When acquiring the mt-cpYFP signal alone, images were captured by exciting alternately at 488 and 405 nm, and collecting the emission at >505 nm. To obtain mt-cpYFP and TMRM signals simultaneously, images were captured by tandem excitation of scan-lines at 488, 405, and 543 nm, and emission collection at 505-530, 505-530, and >560 nm, respectively. To record the mt-cpYFP and NADH signals simultaneously, images were acquired by alternative excitation at 488 nm (confocal) and 720 nm (two-photon), and emission collection at >505 nm and 420-470 nm, respectively. The FAD autofluorescence, which is inversely related to $FADH_2$, was acquired with excitation at 488 nm and emission collection at 500-650 nm. To record the mt-cpYFP and SNARF-1 signals simultaneously, images were acquired by alternative excitation at 488 nm and 405 nm, and emission collection at 505-530, 545-595 (for SNARF-1 S1) and 615-735 (for SNARF-1 S2). The pH change was reported as the S2/S1 ratio. For DCF measurement, the indicator (5 μM) was loaded at 37℃ for 20 min followed by washing 3 times. When clear enrichment in mitochondria was observed, DCF fluorescence imaging was performed by exciting at 488 nm and collecting emission at >500 nm, with a low laser intensity to minimize photochemical reaction of DCF. For mitoSOX measurement, the indicator (5 μM) was loaded at 37°C for 20 min followed by washing 3 times. The mitoSOX fluorescence was reported by exciting at 514 nm and collecting the emission at 559-740 nm. To record the pHTomato signals, images were acquired by excitation at 543 nm and emission collection at 551-690 nm. For grx1-roGFP2 recording, the images were captured by excitation at 405 nm (S1) and 488 nm (S2) and emission collection at 491-531 nm. The fluorescence ratio S1/S2 showed the changes of redox potential.

In typical time-series recordings of mitoflashes, 100 frames of 900×256 (for adult cardiomyocytes) or 512×512 pixels (for neonatal cardiomyocytes or HeLa cells) were collected at 0.10-0.14 μm/pixel in bidirectional scanning mode. For grx1-roGFP2 recording, 150 frames were captured at 30-60 frames/min, and the axial resolution was set to 1.0 μm. All experiments were performed at room temperature (22–26°C) unless specified otherwise.

Proton uncaging from NBA

Cells were loaded with 1 mM NBA as the proton donor, as previously described (3). Photolysis of NBA for proton uncaging was mediated by 405 nm laser illumination at various intensities (0.5% to 3% of full laser power of 15 mW) was applied to whole cells or pre-defined subcellular areas of cardiomyocytes. The photolysis protocol was interleaved with image acquisition with excitation at 488 nm, in a line-by-line (whole-cell uncaging) or frame-by-frame fashion (uncaging in subcellular region of interest).

Background pH measurement

Cytosolic pH was measured with SNARF-1 and mitochondrial pH with mt-EYFP or mt-pHTomato. For SNARF-1 fluorescence, images were acquired by exciting at 488 nm and collecting the emission at >545-595 (S1) and 615-735 (S2). SNARF-1 was localized to both mitochondria and the cytosol, signals from the nuclei were used for cytosolic pH measurements. For mt-EYFP fluorescence, images were captured by exciting at 488 nm and collecting the emission at >500 nm. For pHTomato fluorescence, images were acquired by excitation at 543 nm and emission collection at 551-690 nm.

Image processing and mitoflash analysis

Confocal images were analysed using custom-developed programs written in Interactive Data Language (IDL, ITT). Cell-motion artefacts and background fluorescence changes were corrected by image processing and individual mitoflashes were located with the aid of FlashSniper (4).

Numerical simulation of proton spikes during uncaging

Dynamics between protons (H^+) and pH buffers (B) in the matrix were depicted by the following ODEs:

$$\begin{cases} \dfrac{\mathrm{d}[H^+]}{\mathrm{dt}} = -kon \times (B_{tot} - [HB]) \times [H^+] + koff \times [HB] \\ \dfrac{\mathrm{d}[HB]}{\mathrm{dt}} = kon \times (B_{tot} - [HB]) \times [H^+] - koff \times [HB] \end{cases}$$

where $[H^+]$ refers to the free proton concentration; $[HB]$ the proton-bound buffer concentration; $B_{tot} = 0.026M$ the total buffer concentration (5); $kon = 3 \times 10^{10}\ M^{-1}S^{-1}$ the association rate constant (6, 7); $koff = kon \times 10^{-pKd} S^{-1}$ the dissociation rate constant, and pK_d =7 the dissociation constant (5). For initial conditions of uncaging, we set

$$[H^+](t < 0\ ) = 10^{-8}M$$

$$[H^+](t = 0\ ) = 10^{-4}M$$

$$[HB](t \le 0\ ) = \frac{B_{tot}}{1 + 10^{-pkd+pH(t<0\ )}}$$

Numerical simulation used MATLAB ver. 2012a (The MathWorks) running on a DELL desktop PC with an Intel i7-4790 CPU at 3.60 GHz and 8.0 GB of RAM. The mean life time (τ) of uncaged protons was estimated as the 63% decay time of the proton spike:

$$[H^+](\tau) = [H^+]_{basal} + \frac{1}{e}\left([H^+]_{peak} - [H^+]_{basal}\right)$$

The mean distance of diffusion (l)

$$l = \sqrt{6D_H\tau}$$

Where $D_H = 500\,\mu m^2/s$ refers to diffusion coefficient of protons (6). In this model, buffer capacity can be defined as

$$\frac{\partial[B^-]}{\partial pH} = -\frac{\partial[HB]}{\partial pH} = 2.3B_{tot}\frac{10^{-pKd}}{(10^{-pKd} + [H^+])^2}$$

Using the above parameters we have the buffer power to be 5.0 mM at pH 8.0, in agreement with the value measured experimentally (8).

Statistics

Data are expressed as mean ± SEM. When appropriate, Student's t-test was applied to determine the statistical significance. P<0.05 was considered statistically significant.

Supporting References

1. Cheng, H., W. J. Lederer, and M. B. Cannell. 1993. Calcium sparks: elementary events

underlying excitation-contraction coupling in heart muscle. Science 262:740-744.
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4. Li, K., W. Zhang, H. Fang, W. Xie, J. Liu, M. Zheng, X. Wang, W. Wang, W. Tan, and H. Cheng. 2012. Superoxide flashes reveal novel properties of mitochondrial reactive oxygen species excitability in cardiomyocytes. Biophys J 102:1011-1021.
5. Bazil, J. N., D. A. Beard, and K. C. Vinnakota. 2016. Catalytic Coupling of Oxidative Phosphorylation, ATP Demand, and Reactive Oxygen Species Generation. Biophys J 110:962-971.
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8. Poburko, D., J. Santo-Domingo, and N. Demaurex. 2011. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. J Biol Chem 286:11672-11684.

Figure S1

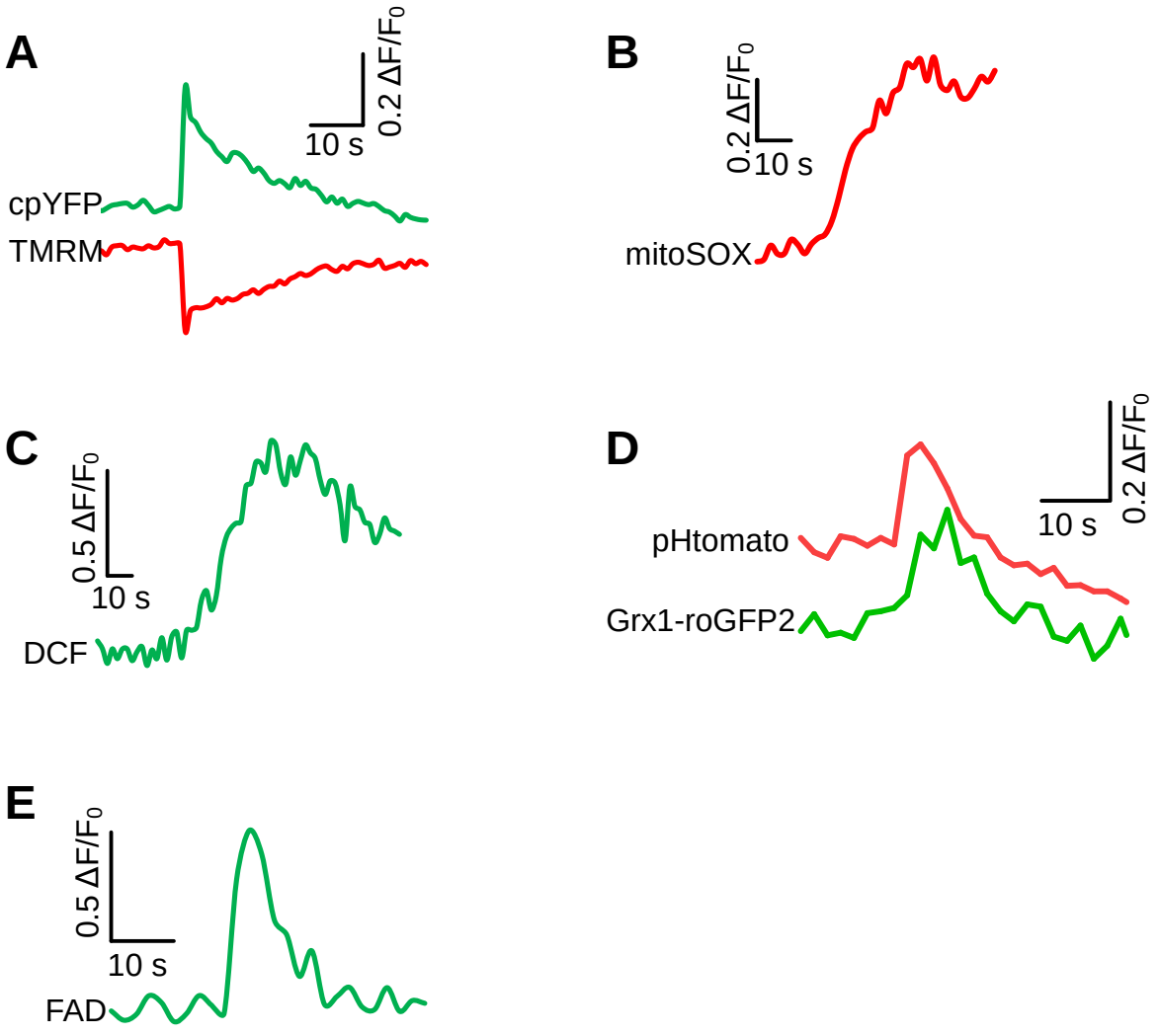

Figure S1. Mitoflashes reported by different indicators in HeLa cells.

Mitoflashes were measured by mt-cpYFP and TMRM (A), mitoSOX (B), DCF (C), pHTomato (D), and grx1-roGFP2 (D), as well as label-free imaging of FAD (E). See also Figure 1. All these mitoflashes occurred spontaneously.

Figure S2

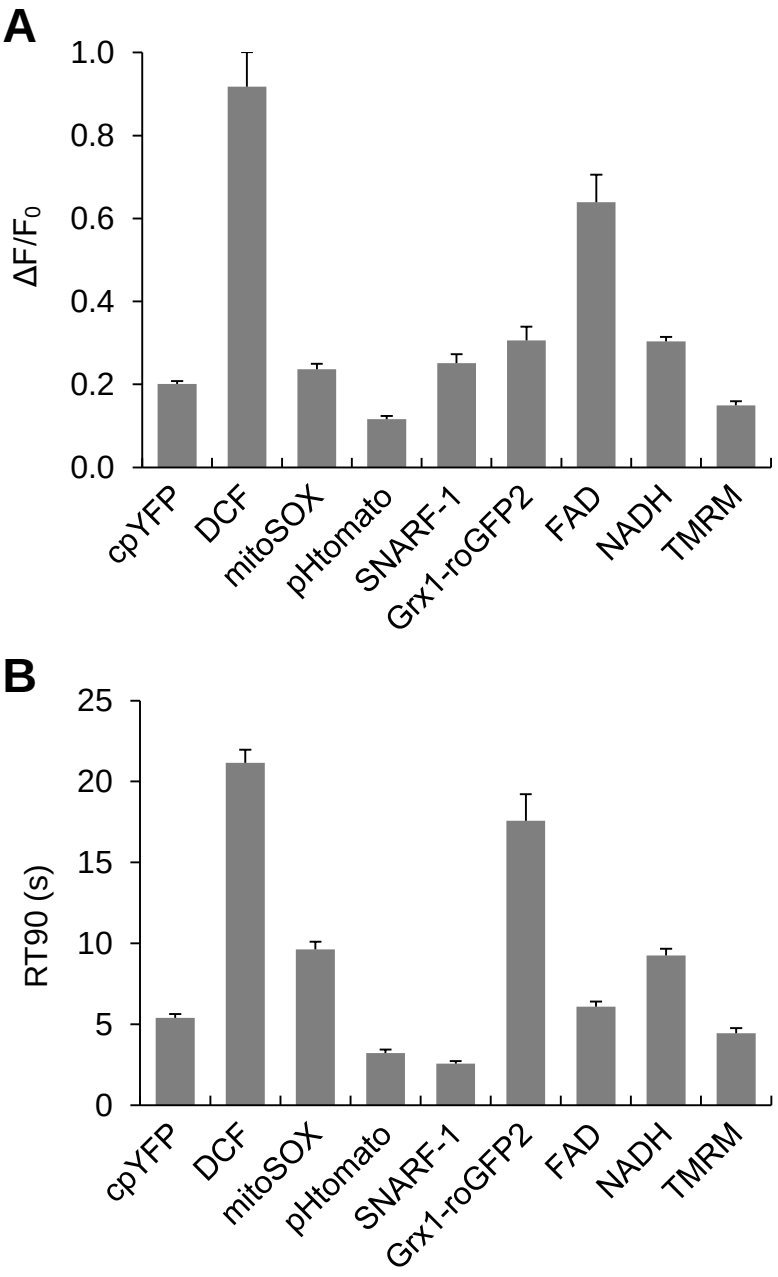
A
1.0
0.8
0.6
0.4
0.2
0.0
ΔF/F0
cpYFP
DCF
mitoSOX
pHtomato
SNARF-1
Grx1-roGFP2
FAD
NADH
TMRM
B
25
20
15
10
5
0
RT90 (s)
cpYFP
DCF
mitoSOX
pHtomato
SNARF-1
Grx1-roGFP2
FAD
NADH
TMRM

Figure S2. Parametric analysis of spontaneous mitoflashes reported by different indicators in adult cardiomyocytes.
(A) Amplitudes of mitoflashes reported by different indicators. Data are reported as mean±SEM of $\Delta F/F_0$, where ΔF refers to the peak change of fluorescence signal and F_0 the basal fluorescence level. (B) Rise time of mitoflashes reported by different indicators. Rise time was measured from the onset to the point at 90% peak amplitude (RT90). The disparities in RT90 may be accounted for by differential kinetics of the underlying signals reported by respective indicators, and by the differences of reaction and deactivation kinetics of these indicators. n = 35-154 spontaneous events for each group.

Figure S3

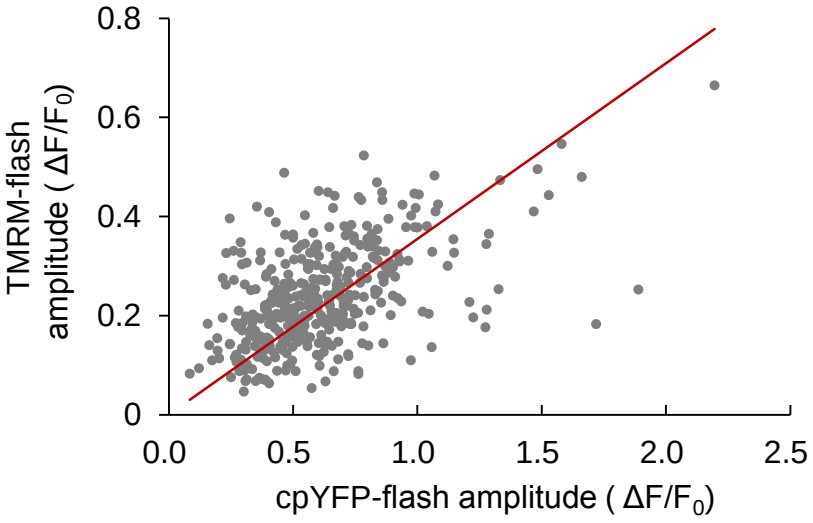

Figure S3. Correlation between the amplitudes of cpYFP- and TMRM-flash events.

Mitoflashes were detected with dual indicators simultaneously in adult cardiomyocytes in the presence of 10 mM pyruvate to increase mitoflash frequency. Line represents a linear fit of the data. r = 0.92, n = 390 events.

Figure S4

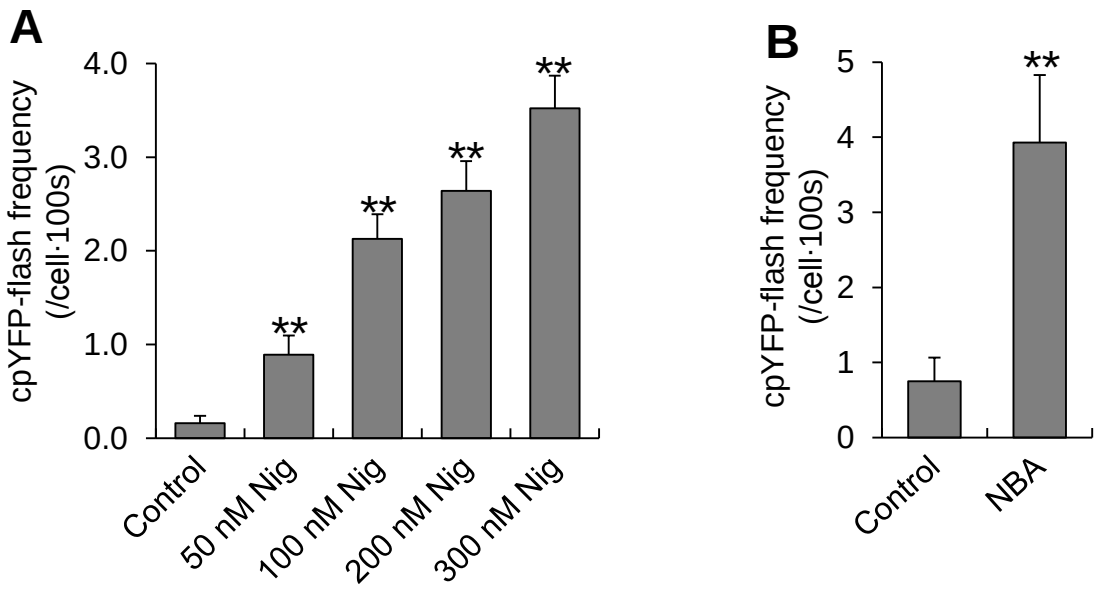

Figure S4. Effects of nigericin and proton uncaging on mitoflash activity in HeLa cells expressing mito-cpYFP.

(A) Concentration-dependent stimulation of mitoflashes by nigericin (Nig**)**. n = 31-48 cells per group. *P <0.05; **P <0.01 *versus* control. (B) Proton uncaging-induced mitoflashes. Cells were treated with 1 mM NBA and 1% 405 nm laser was used for proton uncaging. Cells without NBA treatment were used as the control. n = 8 -14 cells per group. **P <0.01 *versus* control.

Figure S5

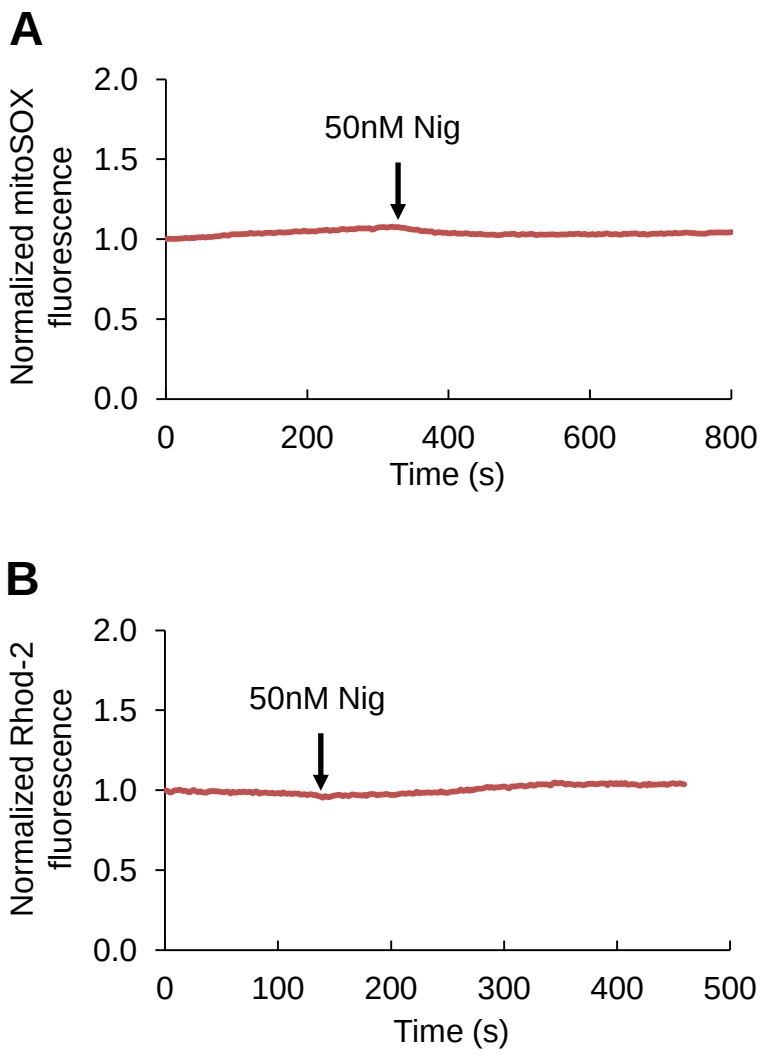

Figure S5. Effects of 50 nM nigericin on mitochondrial ROS and Ca^{2+} in adult cardiomyocytes.

(A) Mitochondrial ROS response measured with mitoSOX. n = 6 cells. (B) Mitochondrial Ca^{2+} response measured with Rhod-2. n = 11 cells. To avoid the interference of cytosolic Rhod-2, cells were saponin-permeabilized and then stimulated with nigericin. Arrows indicate the time of nigericin administration. Data are representative of at least three independent experiments.

Figure S6

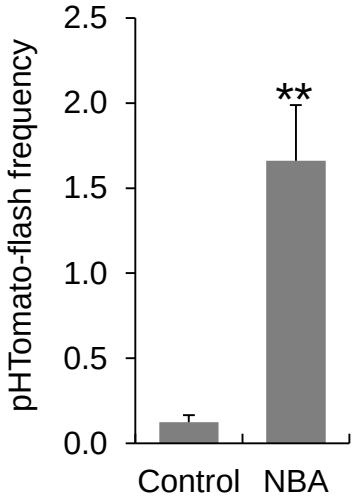

Figure S6. Effects of proton uncaging on pHTomato-reported mitoflashes in adult cardiomyocytes.
Cells were treated with 1 mM NBA and 1.5% 405 nm laser was used for proton uncaging. Cells without NBA treatment were used as the control. n = 11-19 cells per group. **P <0.01 *versus* control.

Figure S7

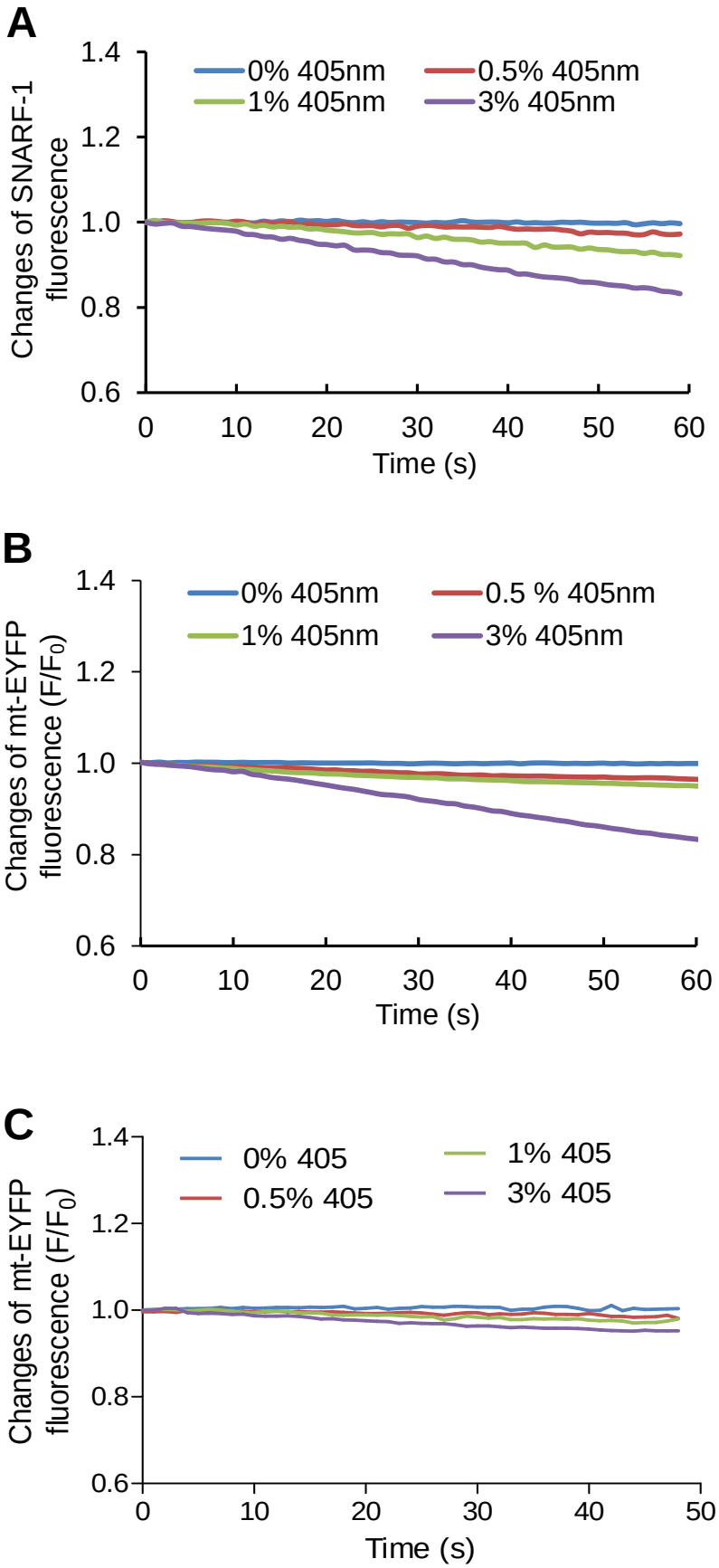
A
1.4
1.2
1.0
0.8
0.6
Changes of SNARF-1 fluorescence
0% 405nm
0.5% 405nm
1% 405nm
3% 405nm
0
10
20
30
40
50
60
Time (s)
B
1.4
1.2
1.0
0.8
0.6
Changes of mt-EYFP fluorescence (F/F0)
0% 405nm
0.5 % 405nm
1% 405nm
3% 405nm
0
10
20
30
40
50
60
Time (s)
C
1.4
1.2
1.0
0.8
0.6
Changes of mt-EYFP fluorescence (F/F0)
0% 405
0.5% 405
1% 405
3% 405
0
10
20
30
40
50
Time (s)

Figure S7. Effects of proton uncaging on cytosolic and mitochondrial pH in adult cardiomyocytes.
NBA-mediated proton uncaging used a 405-nm laser at various intensities (0.5 - 3% of 15 mW). (A) Averaged fluorescence traces of SNARF-1 measuring cytosolic pH in intact cardiomyocytes. n = 6 cells. (B) Averaged fluorescence traces of mt-EYFP measuring mitochondrial pH in intact cardiomyocytes. n = 7 cells. (C) Averaged fluorescence traces of mt-EYFP measuring mitochondrial pH in permeabilized cardiomyocytes. n = 12 cells. Note that background acidosis developed progressively over the entire period of photolysis, evidenced by the decrease of SNARF-1 and mt-EYFP fluorescence signal. This temporal profile is in sharp contrast to that of the mitoflash response illustrated in Figure 4. Note also that cytosolic presence of 20 mM HEPES as pH buffer markedly depressed bulk matrix change in pH during photolysis.

Figure S8

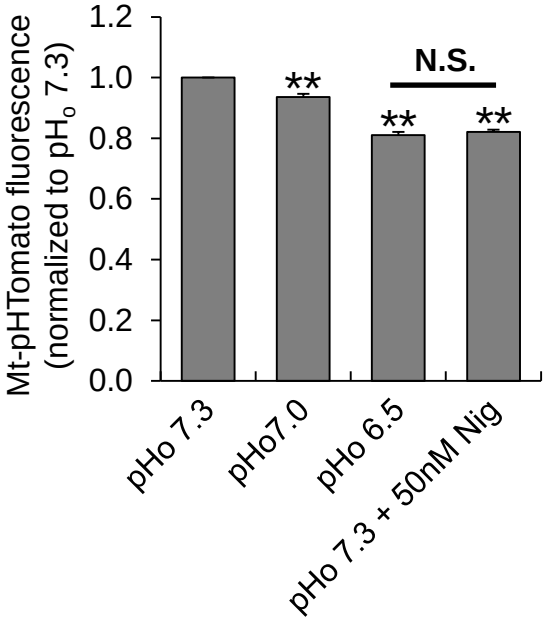

Figure S8. Mitochondrial pH change under various conditions in adult cardiomyocytes. Mitochondrial pH was measured with mt-pHTomato. The decrease of pHTomato fluorescence indicates acidification of mitochondrial matrix. Note that 50 nM nigericin induced a steady pH change that was similar to that at pH_o 6.5. n = 16 cells per group. **P <0.01 *versus* pH_o 7.3 group. N.S., no significance.

Supplementary movie legend:

Movie S1: Mitoflash response to proton uncaging. Arrows indicate the occurrence of mitoflashes. The basal fluorescence level is uniformly elevated upon 405 nm illumination due to the photoconversion effect of cpYFP. Scale bar, 10 μm.