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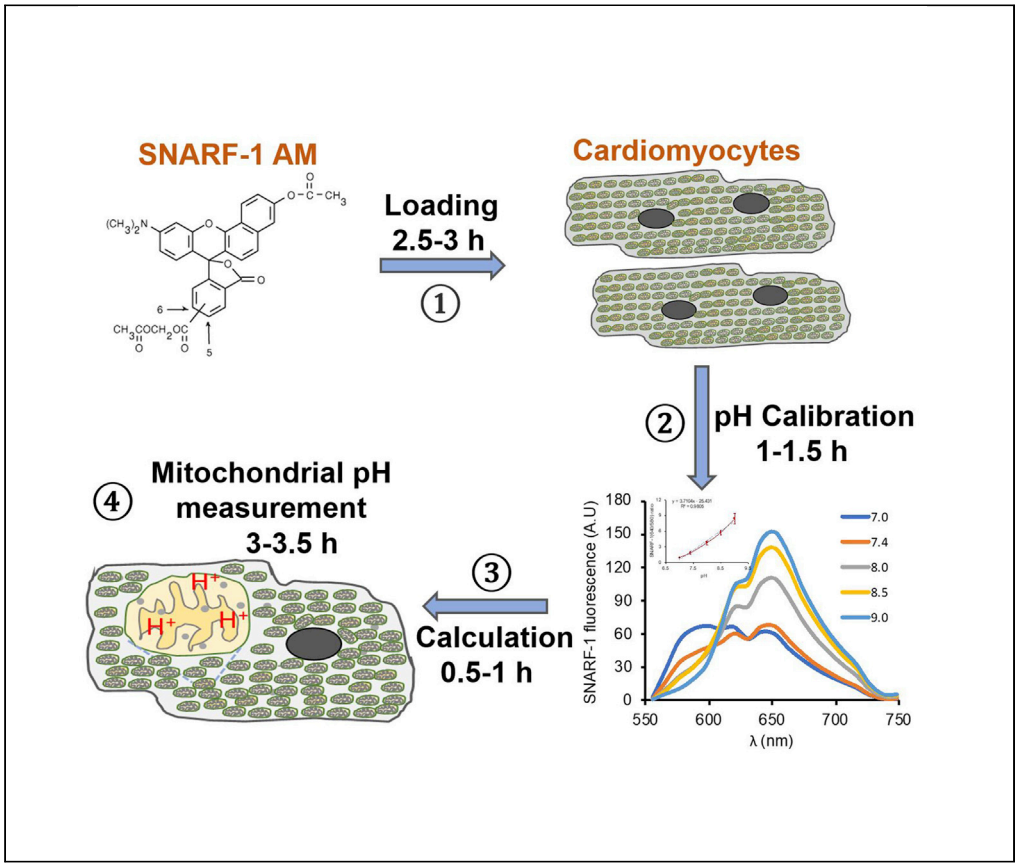
Protocol

Calibration and measurement of mitochondrial pH in intact adult rat cardiomyocytes



Mitochondrial pH is a vital parameter of the mitochondrial environment, which determines the rate of many mitochondrial functions, including metabolism, membrane potential, fate, etc. Abnormal mitochondrial pH is always closely related to the health status of cells. Analyzing mitochondrial pH can serve as a proxy for mitochondrial and cellular function. This protocol describes the use of SNARF-1 AM, a pH-sensitive fluorophore, to measure mitochondrial pH. This protocol details the steps to evaluate mitochondrial pH in live adult cardiomyocytes using confocal microscopy. The protocol can be adapted to other adherent cell types.

Meng Gao, Yuan Qin, Anqi Li, ..., Ying Zhang, Yufei Gao, Guohua Gong

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Highlights

SNARF-1 AM is a pH-sensitive fluorophore for measuring mitochondrial pH

Mitochondrial pH measurements in live cells with confocal microscopy

This protocol uses live cardiomyocytes but can be easily adapted to other adherent cells

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Calibration and measurement of mitochondrial pH in intact adult rat cardiomyocytes

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SUMMARY

Mitochondrial pH is a vital parameter of the mitochondrial environment, which determines the rate of many mitochondrial functions, including metabolism, membrane potential, fate, etc. Abnormal mitochondrial pH is always closely related to the health status of cells. Analyzing mitochondrial pH can serve as a proxy for mitochondrial and cellular function. This protocol describes the use of SNARF-1 AM, a pH-sensitive fluorophore, to measure mitochondrial pH. This protocol details the steps to evaluate mitochondrial pH in live adult cardiomyocytes using confocal microscopy. The protocol can be adapted to other adherent cell types.

For complete details on the use and execution of this protocol, please refer to Wei-LaPierre et al. (2013).

BEFORE YOU BEGIN

Timing: 0.2–2 days

SNARF-1 AM, a derivative of Carboxy SNARF-1, can accumulate into mitochondria. Once inside the matrix, mitochondrial esterases cleave the AM ester to liberate SNARF-1. A warm loading temperature (37°C) favors cytosolic loading, whereas cold temperature (4°C) favors mitochondrial loading in addition to cytosolic loading. At the warm temperature, cytosolic esterases are so active that the AM esters are first cleaved before they can even enter mitochondria. At cold loading temperatures when enzymatic activity is slowed, the fluorophore esters can reach mitochondria before being hydrolyzed, allowing both cytosolic and mitochondrial loading to occur. Then, the anion transporters in the plasma membrane can transport negatively charged, cytosolically localized fluorescent dyes out of cells during incubation.

1. Prepare diameter 25 mm circular coverslips.
   a. Soak coverslips with anhydrous ethanol in a beaker to remove the organic matter on the surface of coverslips.
   b. Discard ethanol and wash coverslips twice with ddH_2O.
   c. Dry the coverslip in the incubator.
   d. Autoclaved coverslips at 121°C for 30 min.

2. Prepare necessary solutions before the pH calibration and measurement. Refer to the key resources table and materials and equipment sections for a complete list of materials and equipment.
3. Prepare freshly isolated or cultured cardiomyocytes according to our step-by-step STAR protocol (Tian et al., 2020) or other protocols before you start the study.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
M199	Sigma-Aldrich	Cat# M2520
L-Glutathione reduced	Sigma-Aldrich	Cat# G6013
L-Carnitine	Sigma-Aldrich	Cat# C0158
Creatine	Sigma-Aldrich	Cat# C3630
Taurine	Sigma-Aldrich	Cat# T8691
$NaHCO_3$	Sigma-Aldrich	Cat# V900182
Insulin-Transferrin-Selenium-X	Thermo Fisher Scientific	Cat# 51500056
Pen/Strep(100×)	Thermo Fisher Scientific	Cat# 10378016
Fetal bovine serum	Thermo Fisher Scientific	Cat# 12483020
Laminin	Thermo Fisher Scientific	Cat# 23017015
$CaCl_2$	Sigma-Aldrich	Cat# V900266
KCl	Sigma-Aldrich	Cat# V900068
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# V900270
$MgCl_2.6H_2O$	Sigma-Aldrich	Cat# V900020
EGTA	Sigma-Aldrich	Cat# E3889
D-Glucose	Sigma-Aldrich	Cat# G8270
HEPES	Sigma-Aldrich	Cat# V900477
Dextrose	Sigma-Aldrich	Cat# G8270
2,3-Butanedionemonoxime	Sigma-Aldrich	Cat# B0753
KOH	Sigma-Aldrich	Cat# 5958
NaOH	Sigma-Aldrich	Cat# S8045
Ethanol absolute	Sigma-Aldrich	Cat# 51976
Ammonium chloride	Sigma-Aldrich	Cat# A9434
FCCP	Sigma-Aldrich	Cat# SML2959
Dimethyl sulfoxide	Sigma-Aldrich	Cat# 276855
SNARF-1 AM acetate	Thermo Fisher Scientific	Cat# C1272
MitoTracker Green	Thermo Fisher Scientific	Cat# M7514
Nigericin	Topscience	Cat# T16323
Experimental models: Organisms/strains		
SD rat	Shanghai SLAC	Cat# SlacSD
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/download.html
Zen	Zeiss	https://www.zeiss.com/microscopy/int/software-cameras.html
Other		
20 mL Syringe	Huanxi Medical	Cat# 66949
Fine-tip forceps	Sangon Biotech	Cat# F519021
Pasteur pipette	NEST	Cat# 318314
6-Well plate	Thermo Fisher Scientific	Cat# 140657
Quick Release Magnetic Chambers	Warner Instruments	Cat# 64-1947
Microscope coverslip	Marienfeld	Cat# AP-0111650
Water bath	YIHENG China	Cat# HWS-12/24
Inverted microscope	Leica	DMi8

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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

Solution preparation

Note: Prepare all solutions using 18.2 Ω MilliQ sterilized H_2O, anhydrous dimethyl sulfoxide (DMSO) or absolute ethanol.

- temperature (20°C–26°C).
- **0.2M EGTA**: Dissolve 7.607 g EGTA in 90 mL ddH_2O, titrate pH to 8.0 with KOH, volume to 100 mL with ddH_2O. Store at 4°C.
- **0.1M 2,3-Butanedione monoxime:** Dissolve 1.011 g 2,3-Butanedione monoxime in 100 mL ddH_2O. Store at -20°C
- **10 mM Nigericin:** Dissolve 14.5 mg Nigericin in 2.0 mL absolute ethanol. Dispense into aliquots and store at -80°C.
- **5 mM SNARF-1 AM acetate:** Add 17.6 μL DMSO to a vial of SNARF-1 AM lyophilized solid (50 μg) and store at -20°C.

Note: SNARF-1 AM acetate should be stored at −20°C, desiccated, and protected from light. Because it is susceptible to hydrolysis, it must be protected from moisture during storage.

- **200 μM MitoTracker Green:** Add 372 μL DMSO to a vial of MitoTracker Green lyophilized solid (50 μg), dispense into aliquots and store at −80°C.

Note: MitoTracker Green is quite sensitive to oxidation, especially in solution, and must be protected from light.

- **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, 500μL, 1 mL) and keep at −20°C for up to six months.
- **500 mM NH_4Cl:** Dissolve 26.745 mg NH_4Cl in 1.0 mL ddH_2O. Prepare freshly for everyday usage.
- **300 μM FCCP:** Dilute 10 μL 10 mM ready-made solution to 300 μM with 323 μL DMSO. Dispense into aliquots and store at −20°C.

⚠ 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	~1000 mL
Total	**n/a**	**1000 mL**

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

Culture medium		
Reagent	Final concentration	Amount/volume
M199 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 (100×)	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 (1M)	138 mM	34.5 mL
KCl (1M)	3.7 mM	0.925 mL
$CaCl_2$ (0.1M)	1 mM	2.5 mL
KH_2PO_4 (0.25M)	1.2 mM	1.2 mL
$MgSO_4$ (100 mM)	1.2 mM	3 mL
HEPES(0.5M)	20 mM	10 mL
Glucose	5 mM	0.6756 g
ddH_2O	n/a	~198.8 mL
Total	**n/a**	**250 mL**

Note: Adjust the pH to 7.4 with 1M KOH, filter with a 0.45 μm bottle top filter, dispense into 50 mL aliquots and store at −20°C.

Calibration solution		
Reagent	Final concentration	Amount/volume
KCl (1M)	140 mM	14 mL
$MgCl_2$ (0.5M)	1 mM	0.2 mL
Dextrose (1 M)	11 mM	1.1 mL
EGTA (0.2M)	2 mM	1 mL
HEPES (0.5M)	12 mM	1.2 mL
2,3-butanedione monoxime (0.1 M)	15 mM	15 mL
Nigericin (10 mM)	10 μM	0.1 mL
ddH_2O	n/a	~76.4 mL
Total	**n/a**	**100 mL**

Note: Solution pH varied from 7.0 to 9.0 (7.0, 7.4, 8.0, 8.5, 9.0) by titration with1M KOH, filter with a 0.45 μm bottle top filter, dispense into 10 mL aliquots and store at −20°C. 2,3-butanedione monoxime was used to prevent cell contracture during the application of the calibrating solutions.

⚠ CRITICAL: Ca^{2+} overload can cause the dysfunction of mitochondrial. EGTA is used to chelate Ca^{2+}.

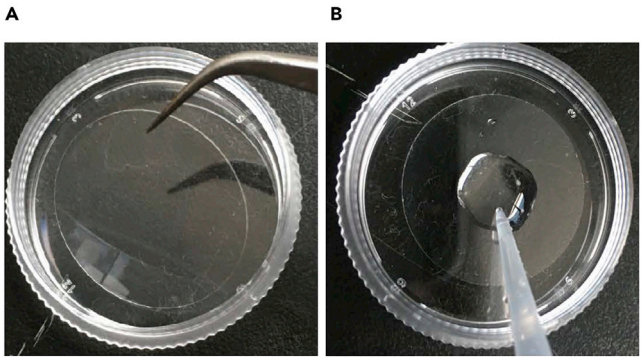

Figure 1. Coverslip coating

(A) Put a 25 mm autoclaved coverslip into 35 mm dish with fine-tip forceps.

(B) Add 100 μL laminin to the center of the coverslip for coating.

STEP-BY-STEP METHOD DETAILS

Coating coverslips

Timing: 1–2 h

Adult cardiomyocytes have poor adhesion. Coating coverslips with laminin can enhance adult cardiomyocyte adhesion.

1. Put an autoclaved 25 mm circular coverslip to a 35 mm dish by a fine-tip forceps (Figure 1A).

   Alternatives: Use the 35 mm glass-bottom dishes.

2. Coat the coverslips with 100 μL 40 μg/mL laminin, carefully spread the laminin over the coverslip using the pipette tip (Figure 1B).

   Alternatives: Add 500 μL laminin on the coverslips, moving plates backward and forward, then right to left to right, let laminin spread over the coverslip.

   Note: This step is not necessary for cell types that have strong adhesion.

3. Gently put the plate into a 37°C incubator for 1–2 h.

Culturing cardiomyocytes

Timing: 2–4 h

4. Count cardiomyocytes (Tian X et al., 2020) using a traditional Hemocytometer, and dilute cells into 2.5×10^5 / mL with M199 culture medium (Figure 2A).

   Note: Appropriate cell number is very important for live-cell imaging. The rod-shaped cardiomyocytes are easy to cross and overlay together. Cell counter may not work on it.

5. 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.

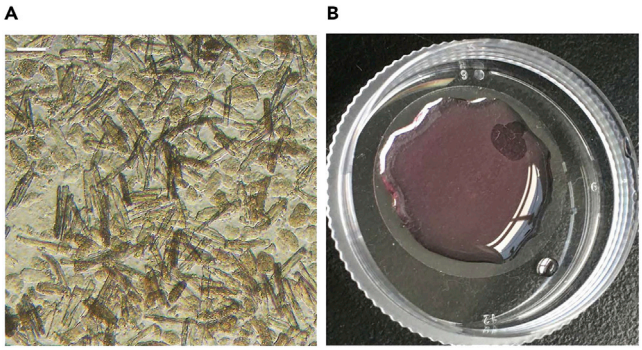

Figure 2. Cardiomyocyte culture
(A) A representative image of collected adult rat cardiomyocytes.
(B)Add 200 μL counted myocytes on a coated coverslip for cultivation. Scar bar= 50 μm.

6. Add 200 μL cardiomyocyte suspension to the center of the coverslip, and the cells will automatically spread on the coverslip (Figure 2B).
7. Gently put the plate into a 37°C, 5% CO_2 incubator, wait for 2–4 h, let cardiomyocytes attached to the coverslip.

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

8. Gently discard the 200 μL medium and add 2 mL fresh M199 culture medium; the non-adherent cells will be discarded, incubate at 37°C, 5% CO_2.

Pause point: The plated cardiomyocytes can wait for up to 72 h to measure pH depends on the different treatments.

SNARF-1 loading

Timing: 2.5–3 h

9. Take the dish out and add 2 μL 5 mM SNARF-1 AM into a well and mix well through gently moving the dish backward and forward 3 times.
10. Incubate cells in media containing 5 μM SNARF-1 AM at 4°C for 30 min (Trollinger, et al., 1997).

Note: Successful load SNARF-1 for measuring mitochondrial pH requires mitochondrial localization of the fluorophore because the membrane-permeable acetoxymethyl (AM) ester form of carboxy SNARF-1 AM is also localized to the cytosol. To distinguish mitochondria, it is better to stain the mitochondria with a mitochondrial-specific fluorescent probe, MitoTracker Green molecular probe.

Alternatives: Incubation of SNARF-1 AM at room temperature for 45 min.

11. Discard the medium with SNARF-1 AM, and equilibrate myocytes with 2 mL KHB solution at room temperature (23°C–26°C) for 1.5 h.
12. Add 1 ul 200 mM MitoTracker Green molecular probe into the dish and mix well through gently moving dish backward and forward 3 times, and incubate at 37°C for 30 min for co-staining mitochondria.

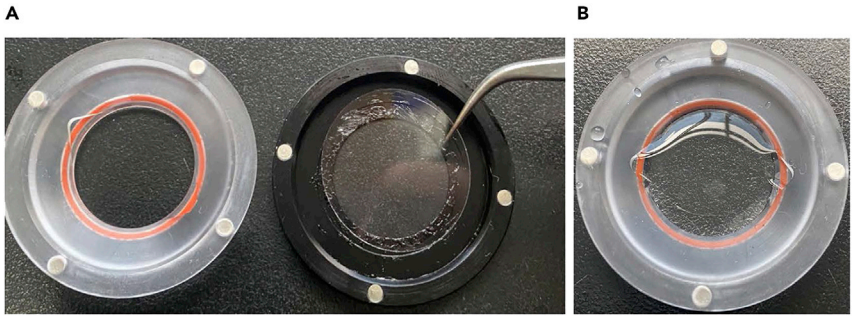

Figure 3. Prepare cell for the confocal image

(A) Put the culture cells to a grease sprayed magnetic image chamber with fin-tip forceps.

(B) The ready myocytes on the imaging chamber.

Note: The anion transporters in the plasma membrane can transport negatively charged, cytosolically localized SNARF-1 AM fluorescent dyes out of cells (Takahashi et al., 2001).

Calibration mitochondrial pH by confocal imaging

Timing: 1–1.5 h

13. 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 3A).
14. Using fine-tip forceps to pick the coverslip up, put it on a Kimwipes cleaning paper to dry the bottom of the coverslip.
15. Place the coverslip into the magnetic bottom of the chamber and put the magnetic top back to fix the coverslip (Figure 3).

Note: To maintain the coverslip's integrity, don't use the fine-tip forceps to press the coverslip. It will make the coverslip broken easily.

16. Add 1 mL pH7.0 calibration solution with a pipette into the chamber and equilibrate 5 min (Figure 3B).

Note: Put the imaging chamber on a Kimwipes cleaning paper to check the leakage.

⚠ CRITICAL: Steps 15 and 16 must be performed quickly (20–40 s) and carefully. Otherwise, adult cardiomyocytes will eventually shrink and die after leaving the solution too long.

17. Add one drop of oil on the 63× objective, place the chamber on the microscope stage above the objective.
18. Find and focus the cell sample in the eyepieces, and move it to the center field of vision.
19. Set the imaging parameters of Zen software, the emission spectra of SNARF-1 AM were collected by excitation at 543 nm using the Lambda scan mode of the Zeiss LSM 880. SNARF-1 AM emission fluorescence was collected at 545–750 nm.Images were acquired at 1024 × 1024 resolution. Gain 450–600; Pinhole 50–200; laser 5%–8%.
20. Discard the calibration solution in chamber on the stage, wash one time for 2 min with 1 mL pH 7.4 calibration solution, add 1 mL pH7.4 calibration solution and equilibrate 5 min to the emission spectra of SNARF-1 AM.
21. Repeat steps 18–20 three times till the emission spectra of SNARF-1 AM in pH8.0, 8.5, and 9.0 are collected.

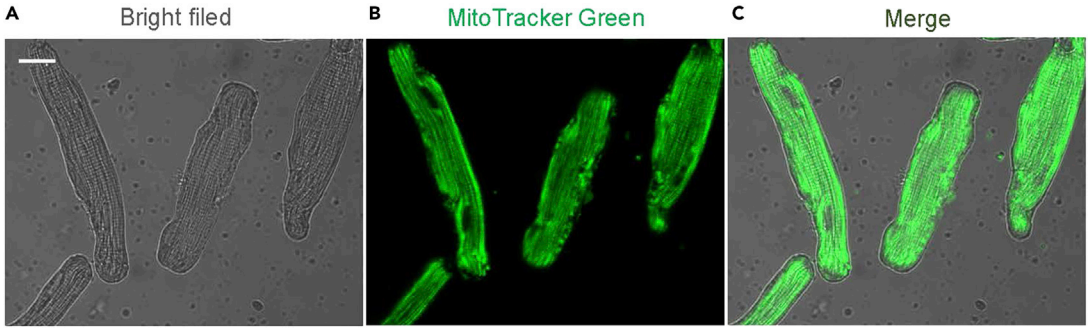

Figure 4. A representative image of 1-day culture cardiomyocytes

(A) Bright filed image of myocytes.

(B) Myocytes loaded with MitoTracker green.

(C) Merged image. Scale bar=20 μm.

Measure mitochondrial pH by confocal imaging

22. Set the imaging parameters of Zen software: Dual excitation images of MitoTracker Green probe and SNARF-1 AM were taken by sequential excitation at 488 nm and 543 nm. MitoTracker Green probe fluorescence was collected at 505–545 nm. SNARF-1 AM fluorescence was collected at 545–600 nm (S1) and >615 nm (S2). Images were acquired at 1024 × 1024 resolution. Gain 450–600; Pinhole 50–200; laser 5%–8%.
23. Set cells in the chamber for imaging follow steps 13–15, add pH7.4 KHB solution.
24. Find and focus the cell sample in the eyepieces, choose only 488 channel to pre-setup relevant imaging parameters by the live scan (Figure 4).
25. Choose the rod cardiomyocytes with a clear mitochondrial pattern to start acquiring 2D images (interval 30 s).
26. After 4th frame is taken, add 10 mM NH_4Cl to the calibration solution and wait automatic image taken.
27. After 14th frame is taken, add 300 nM FCCP to the calibration solution and wait automatic image taken.
28. Stop acquisition after 23th frame is taken and save images.

Analyze images

29. Use the ZEN software to get the S1(580 nm) and S2 (640 nm) intensity of mitochondrial SNARF-1 AM (Figure 5).
    a. Open the Zen software and chose 'ZEN image processing'.
    b. Open the cell image and chose the 'Profile' button (green arrow indicated).
    c. Zoom in the image.
    d. Chose the specific mitochondrion with the definition tool (Red arrow indicated), then the intensity of mitochondria will be displayed in the left panel(Figure 5).
    e. Export these data and calculate the mean of S1 and S2.
30. To calculate the mitochondrial pH, the S1 would be divided by S2 (S2/S1 ratio) and calculated according to the correlated calibration line (Figure 6C).

EXPECTED OUTCOMES

The goal of the method is to analyze mitochondrial pH in intact cardiomyocytes using SNARF-1. Mitochondrial loaded SNARF-1 was colocalized with MitoTracker Green molecular probe (Figure 6A). The lambda emission spectra of SNARF-1 AM showed sensitivity to pH change (Figure 6B). After correlate SNAR-1 640/580 ratio, we got the standard calibration line (Figure 6C). The pH of

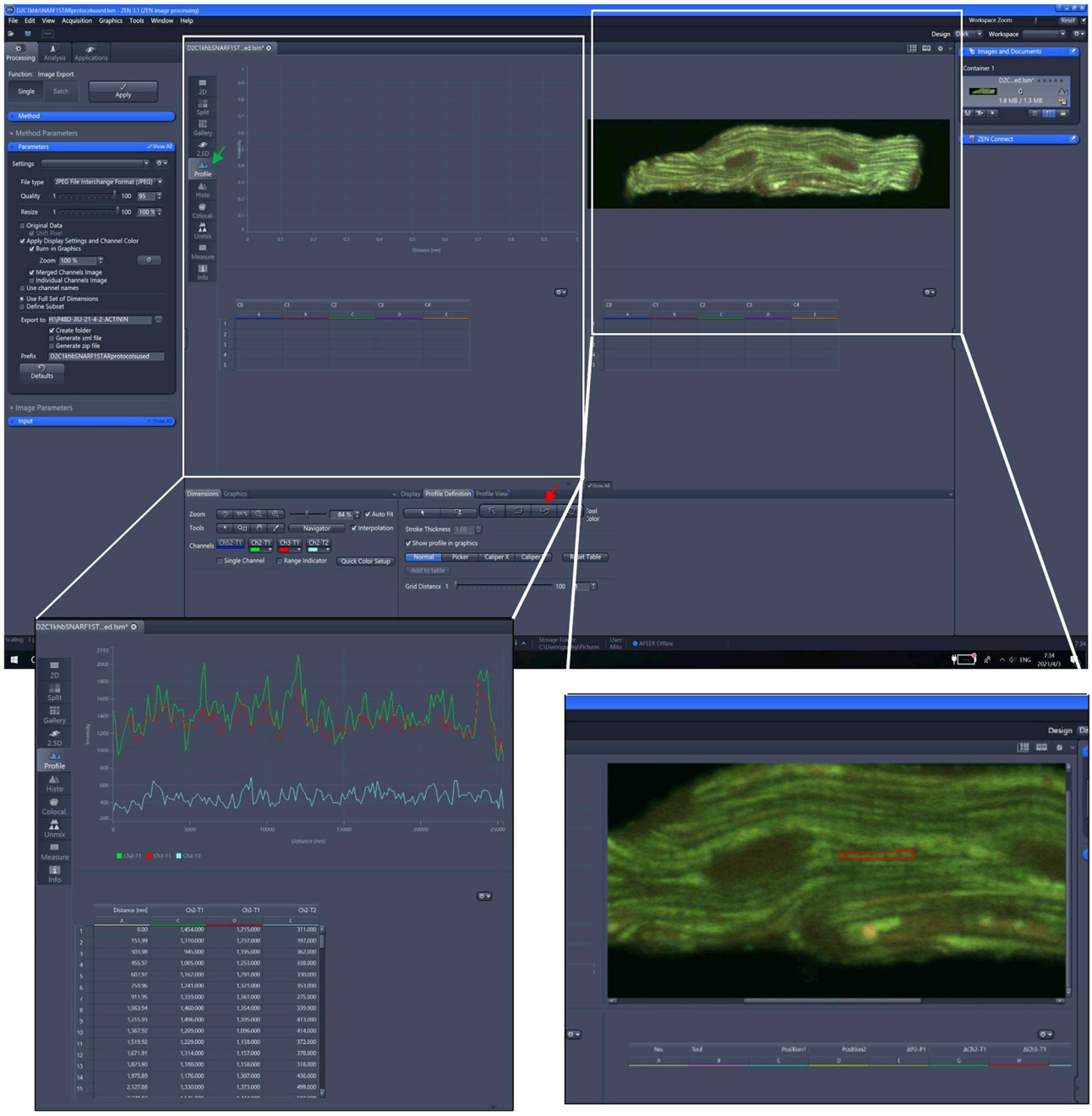

Figure 5. The screenshots for the image analysis

mitochondria is sensitive to the transient alkalinization induced by NH_4Cl and acidification induced by FCCP-triggered respiration uncoupling (Figure 7).

LIMITATIONS

The use of SNARF-1 AM has its drawbacks. Load SNARF-1 AM specific into mitochondria needs more time than load it into the cytosol. If the pH of the mitochondria and the surrounding cytosol are similar, it may be challenging to distinguish mitochondria from the cytosol. This method is not able to separately investigate distinct mitochondrial subpopulations.

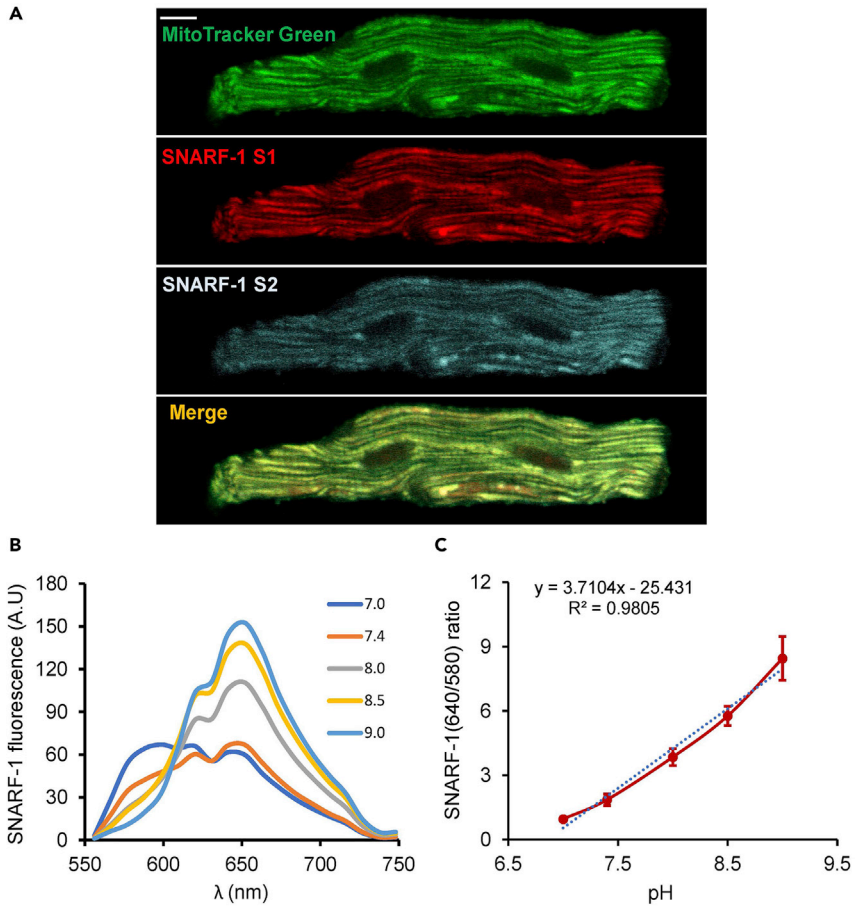

Figure 6. Mitochondrial pH calibration

(A) A representative image of cardiomyocyte loaded with MitoTracker green and SNAR-1.

(B) A representative lambda emission spectra of mitochondrial SNAR-1 during calibration at 543 nm excitation.

(C) Correlated calibration line of mitochondrial SNARF-1. Scale bar=10 μm.

TROUBLESHOOTING

Problem 1

The SNARF-1 is not well loaded into mitochondria.

Potential solutions

The ultimate intracellular distribution of SNARF-1 AM is dependent on the activity of cytosolic and organelle esterases relative to the rate of uptake of the AM form of the dye into the cytosol and organelles. To promote mitochondrial uptake, cells can be loaded with SNARF-1 AM at a higher temperature (37°C) for a longer time (3–4 h).

Problem 2

Mitochondria pattern with SNARF-1 AM is not clear

Potential solutions

Incubate SNARF-1 AM at 4°C for 45–90 min, and then incubate cells with KHB 3–4 h promotes the loss of cytosolically localized SNARF-1 AM dyes but retains mitochondrial accumulated probes intact.

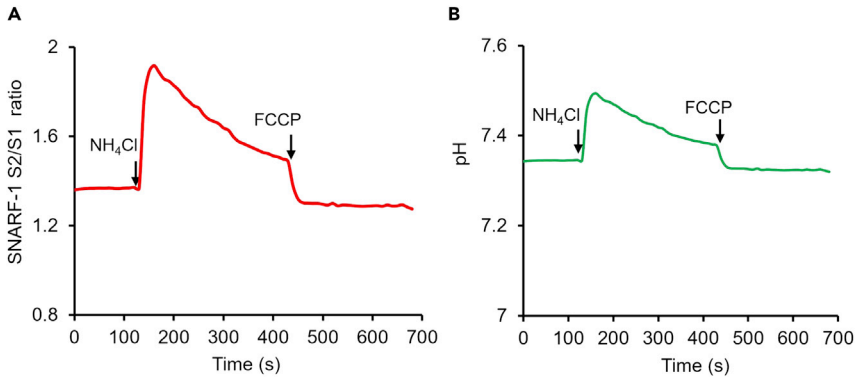

Figure 7. An example of mitochondrial pH measurement

(A) A representative trace of mitochondrial SNARF-1 ration change triggered by reagents.

(B) A representative trace of mitochondrial pH change is calculated from the SNARF-1 ratio.

Problem 3

Cell contraction affects imaging.

Potential solutions

Before applying the calibration solution, incubate the myocytes in the KHB solution containing 2 mM EGTA and no added Ca^{2+} for ~2 min to remove extracellular Ca^{2+}.

Problem 4

Solution leakage of chamber

Potential solutions

Apply more high vacuum grease to the pedestal of the Magnetic bottom and rigorously dry the bottom of the coverslip with a Kimwipes cleaning paper.

Problem 5

FCCP does not trigger the change of mitochondrial pH

Potential solutions

Add more FCCP or prepare the new stock solution of FCCP.

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

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

G.G. and Y.Q. conceived, designed, and supervised the project. M.G. and Y.Q. conducted most experiments and performed data analysis. H.L. maintained the Clark electrode. L.C., Y.Z., and Y.G. prepared solutions. A.L. and B.L. provided valuable suggestions. G.G. and M.G. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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