

Video Article

Detection of Mitochondria Membrane Potential to Study CLIC4 Knockdown-induced HN4 Cell Apoptosis *In Vitro*

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Abstract

Depletion of the mitochondrial membrane potential (MMP, $\Delta\Psi_m$) is considered the earliest event in the apoptotic cascade. It even occurs ahead of nuclear apoptotic characteristics, including chromatin condensation and DNA breakage. Once the MMP collapses, cell apoptosis will initiate irreversibly. A series of lipophilic cationic dyes can pass through the cell membrane and aggregate inside the matrix of mitochondrion, and serve as fluorescence marker to evaluate MMP change. As one of the six members of the Cl⁻ intracellular channel (CLIC) family, CLIC4 participates in the cell apoptotic process mainly through the mitochondrial pathway. Here we describe a detailed protocol to measure MMP via monitoring the fluorescence fluctuation of Rhodamine 123 (Rh123), through which we study apoptosis induced by CLIC4 knockdown. We discuss the advantages and limitations of the application of confocal laser scanning and normal fluorescence microscope in detail, and also compare it with other methods.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56317/>

Introduction

Rh123 is a cationic fluorescence dye, which serves as an indicator for transmembrane potential. Rh123 is capable of penetrating the cell membrane and entering the mitochondrial matrix depending on the potential difference of inside and outside the membrane¹. Apoptosis leads to damage of mitochondrial membrane integrity. The mitochondrion permeability transition pore (MPTP) will open and lead to collapse of the MMP, which in turn results in the release of Rh123 to the outside of the mitochondria. Finally, stronger green fluorescence signal will be detected under fluorescence microscope. It is well documented that depletion of the MMP and elevated membrane permeability are early signs of cell apoptosis². Therefore, Rh123 may be applied to the detection of MMP change and the occurrence of cell apoptosis.

As the 6th most common carcinoma in the world, head and neck cancer severely deteriorates a person's health³. Although many approaches were developed in recent years, clinical outcome of treatment for patients suffering from head and neck squamous cell carcinoma (HNSCC) is still not ideal⁴. Exploring new therapeutic methods can improve the treatment for HNSCC⁵. Ion channels involving numerous biological processes display an important role in the development of different cancers⁶. Partial or total participation of Cl⁻ channels are highly involved in various properties of neoplastic transformation including active migration, high rate of proliferation and invasiveness. In light of this, the CLIC, a novel protein family, has been listed as a promising class of therapeutic targets for cancer treatment^{6,7}. Recent studies have revealed that members of the CLIC family including CLIC1, CLIC4, and CLIC5, localize to cardiac mitochondrial and the reactive oxygen species (ROS) level is upregulated by CLIC5, indicating the functional role of mitochondrial-located Cl⁻ channels in the apoptotic response⁸. CLIC4, one members of the CLIC family (also known as mtCLIC, P64H1, and RS43), has been most extensively studied for its apoptotic regulation properties in cancer cells and subcellular location including Golgi, endoplasmic reticulum, and mitochondrion in human keratinocytes^{7,9,10}. The expression profile of CLIC4 was regulated by tumor necrosis factor- α (TNF- α), P53, and external stimulus. Overexpression and downregulation of CLIC4 trigger an apoptotic response mainly through the mitochondrial pathway accompanied with the imbalance of Bcl-2 family members, activation of caspase cascade, and release of cytochrome C^{11,12,13}. Therefore, MMP measurement is crucial to explore CLIC4-related apoptosis, and Rh123 serves as an ideal fluorescence indicator.

The present study describes a detailed protocol for the detection of MMP to study CLIC4 knockdown-induced apoptosis in HN4 cells. Rh123 is used as a fluorescence probe to observe the change of the MMP. Under common fluorescence microscope and confocal laser scanning fluorescence microscope, the real-time fluctuation of the MMP may be resolved. We discuss the advantages and limitations of the application of confocal laser scanning fluorescence microscope in detail, and also compare it with other methods. This protocol also can be applied to other apoptosis-related studies.

Protocol

1. Cell Culture and Transfection

1. Cell culture

Note: HN4, an HNSCC cell line, was derived from patients with HNSCC¹⁴.

1. Culture HN4 cells in Dulbecco's modified Eagle medium (DMEM, 4.5 g/L glucose) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Incubate cells at 37 °C with 5% CO₂.

2. Cell transfection

1. One day before transfection, plate 5×10^5 cells in 2 mL/well of culture medium without antibiotics in 6-well plates.
2. Dilute 100 pmol CLIC4 siRNA or scrambled siRNA in 250 µL of reduced serum media, gently. Dilute 5 µL liposome reagent in 250 µL of reduced serum media and incubate for 5 min. Combine the diluted siRNA with the diluted liposome reagent, mix gently, and incubate for 20 min at room temperature.
3. Add the mixture to each well containing cells and medium. Mix gently by rocking the plate back-and-forth. Incubate the cells at 37 °C in a CO₂ incubator for 24 h before proceeding with the following Rh123 labeling procedure. Replace the medium with normal growth medium 4 h after transfection.

2. Rh123 Labeling

Note: Rh123 was utilized to measure the MMP in HN4 cells¹.

1. After CLIC4 siRNA treatment (Section 1), use HN4 cells in 6-well plates for the following treatment. The amount of HN4 cells in each well is enough to repeat the experiment 10 times on coverslips.
2. Use tweezers to put circular coverslips on the bottom of new 12-well plates. The number of plates is set according to the experimental design (**Figure 1A**).
3. Put 150 µL of 100 µg/mL polylysine on the middle of every coverslip inside the 12-well plates and wait 2 min. Pay attention to not put polylysine outside the coverslips. Then remove the polylysine by pipettor thoroughly (**Figure 1B**).
4. Remove the culture medium of the HN4 cells inside the dish and add 1 mL 0.25% trypsin to detach the cells. After 6 min, add 2 mL culture medium to neutralize the trypsin. Gently mix the HN4 cells by pipettor for 5 times and seed $2 - 6 \times 10^4$ cells on the circular coverslips. Place in an incubator at 37 °C with 5% CO₂ (**Figure 1C**).
5. After 8 h incubation, incubate the HN4 cells with 2 µmol/L Rh123 for 40 min at 37 °C. Gently mix the medium up-and-down several times to ensure even distribution of Rh123 in the medium (**Figure 1D**).
6. Prepare enough normal physiological saline solution (NPSS); to make NPSS (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. Preheat to 37 °C in a water bath.
7. Use tweezers to remove the coverslips and wash the remaining liquid via briefly placing the coverslips into the preheated NPBS buffer (**Figure 1E**).
8. Place the coverslips on the bottom of the 500 µL stainless steel chamber (see **Table of Materials**) with a replaceable 25 mm glass coverslip bottom sealed in place by an o-ring. Fix it by tightening the lid of the chamber (**Figure 1E**).
9. Add 450 µL preheated NPSS buffer to the assembled chamber and put the bath under the visual field of the fluorescence microscope or confocal laser scanning fluorescence microscope (**Figure 1E**).

3. Detection of the MMP

1. Common fluorescence microscope

1. Turn on the fluorescence microscope following the manufacturer's instructions.
NOTE: Common fluorescence microscope was accomplished with a mercury lamp fiberoptic light source, a FITC filter set for Rh123 (480/40 excitation filter, 505 LP dichroic mirror and 535/40 emission filter) at room temperature. 20X objective was used to perform live-cell imaging.
2. Open the imaging system software and select the "New" button from the command bar to begin a new experiment (**Figure 2A**).
3. Adjust the visual field and focus to find the location of the cells in white light.
4. Turn off the light and push the button to close white light. Click the "Focus" button from the Command bar and select "Start Focusing" to switch on the fluorescence. Find the location of the cells again via microscope with a 507 nm excitation and 529 nm long pass emission wavelength (Set before the experiment). Adjust the visual field and focus again if needed (**Figure 2B**).
5. Select a visual field containing only 20 to 30 separated HN4 cells. The change of intracellular fluorescence signal for each cell will be recorded accurately.
6. Once a clear visual field is achieved, click "Close Focusing" from the Focus bar. Click "Acq One" from the Command Bar to acquire one set of images. Click "Regions" from the Command Bar and click the "OK" button to edit measurement regions.
NOTE: From the Edit Region bar, different region shapes can be used depending on the study.
7. To fit for different shapes of individual cells, select the "Trace Region" tool and circle the region of one single cell and double click when finished. Repeat the procedure until all cells are circled (**Figure 2C**). Click "Done" and select "Save Images" to find the saved location.
8. Click "Zeroclock" and quickly push F4 to start monitoring the change of fluorescence intensity. Record the real-time change of fluorescence intensity once every 5 s for 5 min (**Figure 2D**).
9. When the baseline becomes stable, add 50 µL NPSS mixed with 0.5 µL of 100 mmol/L ATP to the chamber via pipettor. Remember to not touch the chamber to avoid removing the visual field (**Figure 2E**).

NOTE: The fluorescence image will be recorded for 20 min and then the experiment can end via manual operation. The time duration is routinely settled for 20 min in order to observe the entire fluorescence change. However, manual operation to stop capture is applied when unexpected factors influence the stability of the curve or when the entire process takes less than 20 min.

2. Confocal laser scanning fluorescence microscope

1. Turn on the confocal laser scanning fluorescence microscope following the manufacturer's instructions, and open the bundled software. NOTE: Here, Rh123 was excited by an Argon laser set at 488 nm and the emitted signal was recorded over the range of 545 - 700 nm via application of the TD488/543/633 filter.
 2. Click "Objective" under the "Acquire" menu to select 63X/1.4 N.A. oil objective lens. Observe the specimen and find a clear visual field under the light field.
 3. Push the "TL/IL" button to switch to fluorescence mode and select the correct fluorescence filters to find the location of the cell under darkness via microscope. Push "SHUTTER" to protect the specimen when observation is finished.
 4. Under the "Acquire" menu, adjust suitable PMT channel and click "Achieve" to activate the settled optical path (**Figure 3A**). Click on the "Configuration" menu and choose "Laser" to determine the needed laser type. "Argon" is recommended for this protocol (**Figure 3B**).
 5. Click "Live" to get real-time image and make sure the visual field contains only 20 to 30 separated HN4 cells. The change of intracellular fluorescence signal for each cell will be recorded accurately.
 6. Select "xyt" in Acquisition Mode in the "Acquisition" submenu under the "Acquire" menu (**Figure 3C**). Set 5 s and 20 min for time interval and duration, respectively. Click "Apply" when all parameters are settled.
 7. Under the "Quantify" menu, use the "Draw Polyline" tool to circle the recording area of each cell. Select "Line Profile" and choose "Start" to conduct observation. Record the real-time change of fluorescence intensity for 5 min (**Figure 3D - E**).
 8. When the baseline becomes stable, add 50 μ L NPSS mixed with 0.5 μ L of 100 mmol/L ATP to the chamber via pipettor. Remember to not touch the chamber to avoid removing the visual field.
- NOTE: The fluorescence image will be recorded for 20 min and then the experiment is completed.

4. Statistical Analysis

1. Common fluorescence microscope

1. Turn on the fluorescence microscope routinely following the operator's manual. Open the imaging system software and select the "Open" button from the command bar to open a saved experiment. Click "Regions" from the 'Command Bar' and click "OK" button to edit measurement regions.
 2. Select the "Trace region" tool and circle the region of one single cell and double click when done. Repeat the procedure until all cells have been circled. Click "Done" and select the "F4: forward" button to obtain a trace showing fluorescence intensity.
 3. Once finished, click the down arrow button located on the lower right corner of the graph and click "Show Graph Data" (**Figure 2F**). Click "OK" twice and choose the "Log Data" button to open the interface of data processing software. Click "Log Data" again and find the records of real time fluctuation of fluorescence intensity appear on the spreadsheet.
- NOTE: For each cell, changes in MMP were displayed as the ratio of fluorescence relative to the intensity before the application of ATP or TG (F1/F0). Each fluorescence intensity data were the average of 20 - 30 cells. Data for Rh123 are presented as mean $\pm$ SE. Results from 2 groups were tested by the two-tailed independent *t* test and *P* value <0.05 was considered as statistical significance. Statistical analysis software was applied to conduct the statistical analyses in this experiment.

2. Confocal laser scanning fluorescence microscope

1. Turn on the confocal laser microscope and log into the bundled software following the manufacturer's instructions.
2. Select "Fire" to open a recorded experiment.
3. Select the "Stack profile" tool under the "Quantify" menu, choose "All in one" from "Sort charts by Channels and ROIs."
4. Use the "Draw polygon" tool to circle cell regions for observation.
5. Select the "Graph" menu and click the right mouse button, and choose "Export to excel" to add the values of fluorescent intensity to a spreadsheet. The following analysis is consistent with normal fluorescence microscopes (**Figure 3F**).

Representative Results

In the present study, Rh123 was applied to detect the MMP. Initially, HN4 cells were cultured for the following fluorescence staining experiments. Tweezers were used to put circular coverslips on the bottom of 6-well plates (**Figure 1A**). The coverslips were coated with polylysine for 5 min and then the polylysine removed via pipettor (**Figure 1B**). Then HN4 cells were trypsinized and seeded on the coverslips placed in the bottom of 6-well plates. Next, the appropriate amount of Rh123 was added and mixed well (**Figure 1C-D**). After washing with the preheated NPSS, the coverslip was assembled into the viewing chamber. The appropriate amount of NPSS was added for the following experiment (**Figure 1E**).

After the procedure of Rh123 staining, both the common fluorescence microscope and confocal microscope were used to record the real-time Rh123 fluorescence with different steps and software. For the common fluorescence microscope, the imaging software was turned on to create a new experiment. The shutter was opened to show the green fluorescence light and the focus and location were adjusted to obtain an appropriate visual field. After taking a cell image, the shape trace tools were used to draw the region of every single cell and then record the fluorescence change. Once the baseline became stable, ATP was added into the chamber to trigger mitochondrial membrane depolarization. In the experiment of confocal laser scanning fluorescence microscope, the bundled software was opened and a suitable optical path was set. After the argon light source was chosen, we adjusted the focus and position of the chamber to achieve a clear visual of the cell shapes on screen. The "xyt" scan mode was selected and the "Draw polygon" tool was applied to circle the cell region to record the fluorescence change within every single cell. To analyze the raw data, F0 was the value of fluorescence intensity before ATP application and F1 was the maximum value of fluorescence intensity after ATP application. F1/F0 was used to assess the mitochondrial membrane depolarization (**Figure 2** and **Figure 3**). From **Figure 4B** and **Figure 5B**, the levels of fluorescent intensity were elevated following the treatment of ATP and decreased back to the baseline after about 10 min. **Figure 4C** and **Figure 5C** clearly demonstrate that the peak of fluorescence intensity of Rh123 was higher in the group under the CLIC4 siRNA treatment than that in control group. **Figure 4A** and **Figure 5A** show representative images of the fluorescent change in each stage. Compared with the common fluorescence microscope, the confocal microscope obtained a higher quality of image, showing cell nucleus and mitochondria. However, when comparing the data from the two methods, no significant differences were found. Results from fluorescence and confocal microscope demonstrated that knockdown of CLIC4 enhanced ATP-induced mitochondrial membrane depolarization in HN4 cells (**Figure 4** and **Figure 5**).

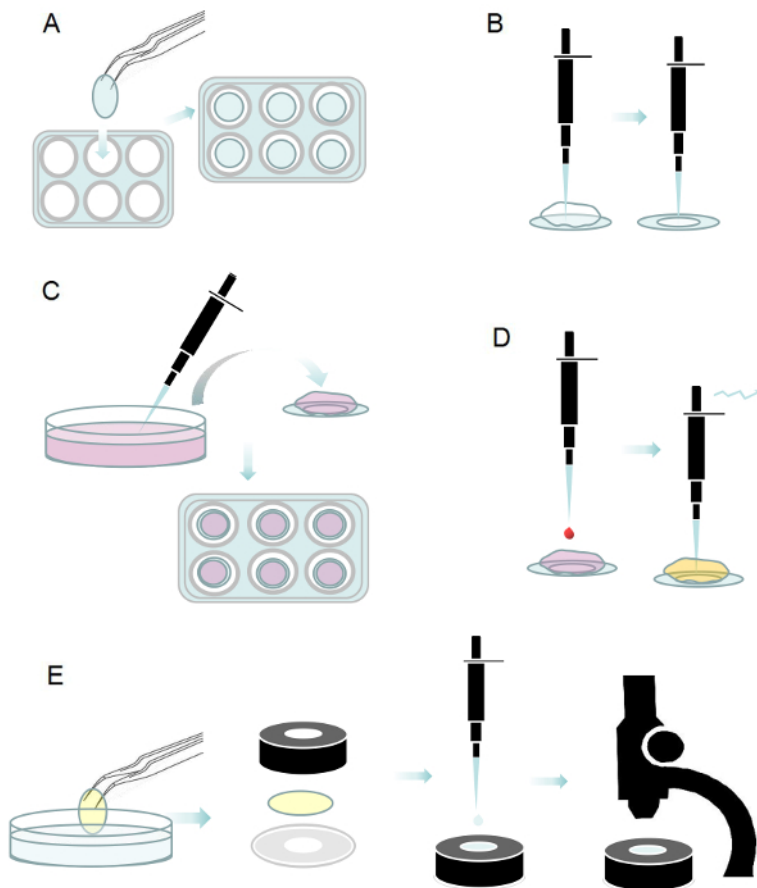


Figure 1. Rh123 labeling. (A) Tweezers were used to put circular coverslips on the bottom of 6-well plates. (B) Coating coverslips with polylysine via pipettor for 5 min and finally suction back into pipettor to remove the polylysine. (C) HN4 cells were seeded on the coverslips on the bottom of 6-well plates. (D) Adding a suitable amount of Rh123 and mixing well. (E) Washing the coverslips with preheated NPSS and assembling the chamber with a suitable amount of NPSS for the following experiment. [Please click here to view a larger version of this figure.](#)

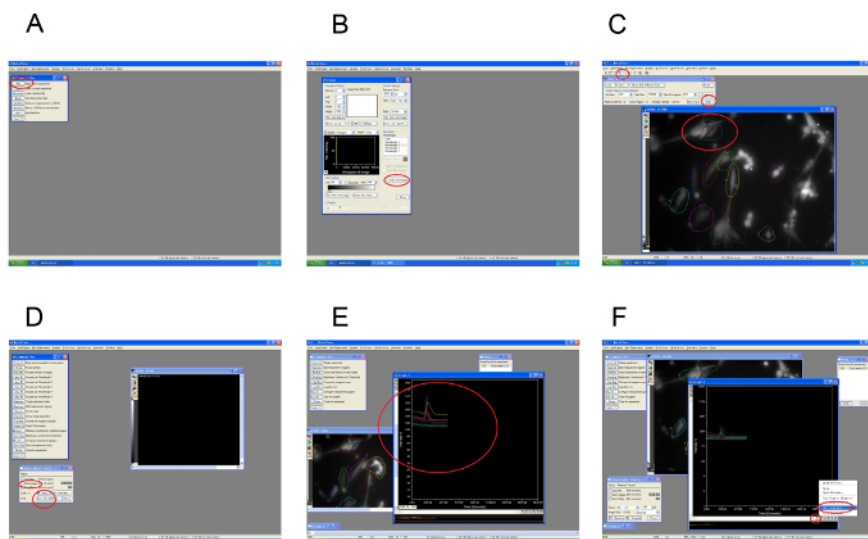


Figure 2. Steps to monitor real-time fluctuation of Rh123 fluorescence by common fluorescence microscope. (A) Open a new experiment. (B) Start focusing and choose a suitable visual field under fluorescence. (C) Using the "Regions" tool from the Command Bar to edit observation regions. (D) Click "Zero clock" and quickly push F4 to start monitoring the change of fluorescence intensity. (E) Adding ATP (100 $\mu\text{mol/L}$) to trigger mitochondrial membrane depolarization after the baseline becomes stable. (F) Once the experiment is finished, click the down arrow located on the lower right corner of the graph to export data. [Please click here to view a larger version of this figure.](#)

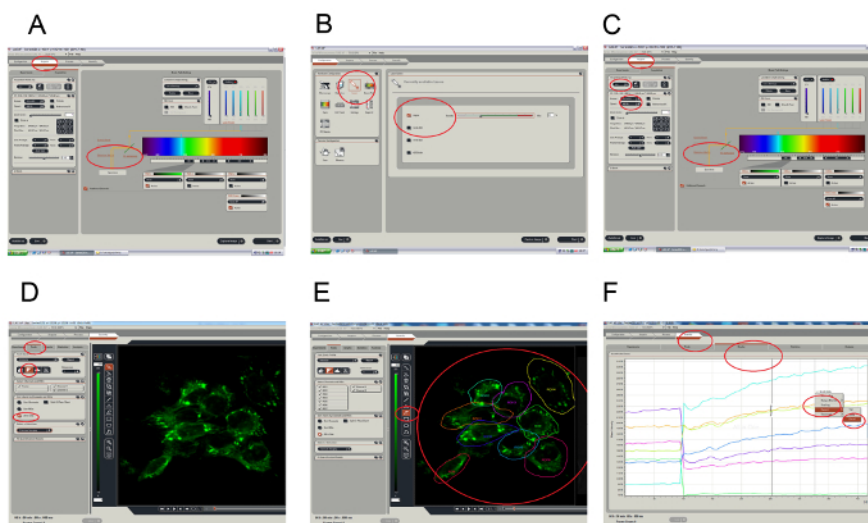


Figure 3. Steps to monitor real-time fluctuation of Rh123 fluorescence by confocal laser scanning fluorescence microscope. (A) Select the PMT channel and set a suitable wavelength range under the "Acquire" menu. (B) Select the correct laser type from "Current available laser" and set its power. (C) Choose the "xyt" observing mode and set a series of recording parameters. (D) Choose the tools "Stack Profile" and "All in One" from "Sort charts by Channels and ROIs." (E) Use the tool "Draw polygon" to circle the cell region for the real-time data. (F) Export the values of fluorescent intensity to data processing software. [Please click here to view a larger version of this figure.](#)

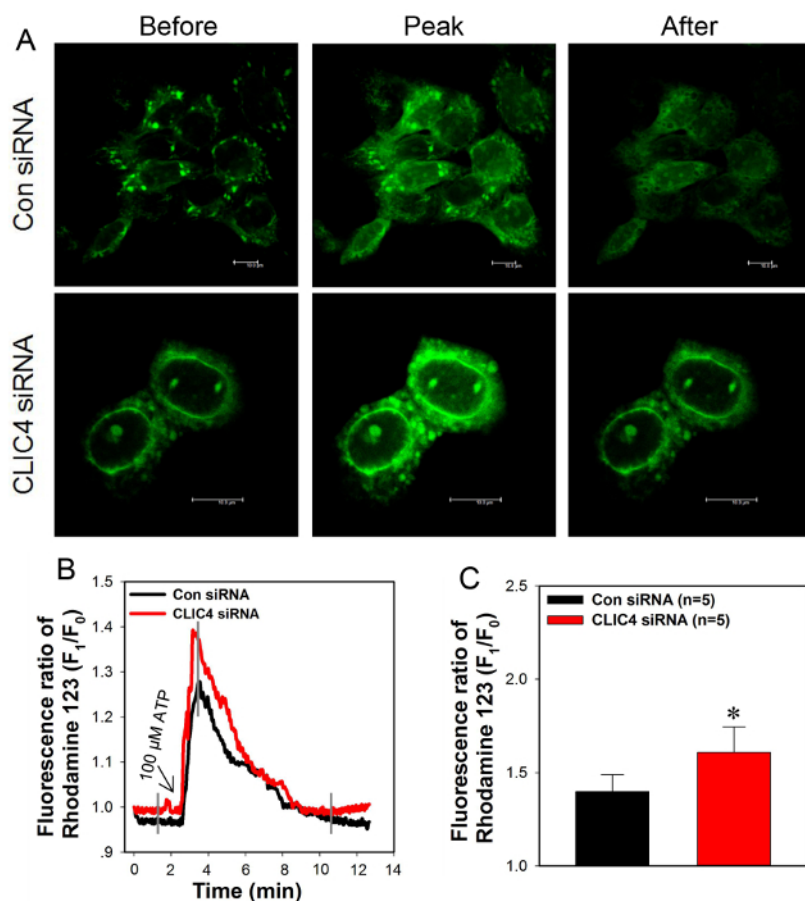


Figure 4. Effect of CLIC4 knockdown on ATP-induced mitochondrial membrane depolarization resolved by common fluorescence microscope in HN4 cells. (A) Representative images of fluorescence fluctuation for Rh123. Representative traces (B) and summarized data (C) showing ATP (100 μmol/L)-induced changes in the HN4 cell MMP. The change in the membrane potential was indicated by the ratio of the fluorescence intensity before and after the application of ATP. The ratio increase represents the membrane potential depolarization. The HN4 cells were transfected with CLIC4siRNA (CLIC4 siRNA) or scrambled siRNA (Con siRNA). Values are shown as the mean ± SEM. n = 5. * P < 0.05 vs. control (Con siRNA) group. [Please click here to view a larger version of this figure.](#)

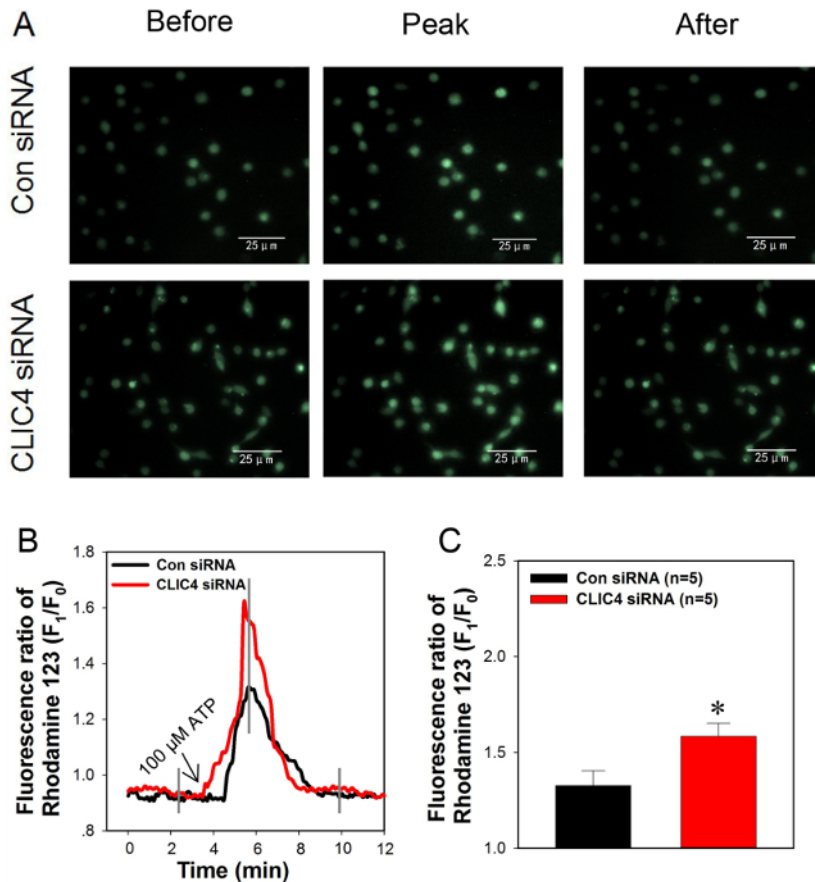


Figure 5. Effect of CLIC4 knockdown on ATP-induced mitochondrial membrane depolarization resolved by confocal laser scanning fluorescence microscope in HN4 cells. (A) Representative images of fluorescence fluctuation for Rh123. Representative traces (B) and summarized data (C) showing ATP (100 μ mol/L)-induced changes in the HN4 cell MMP. The change in the membrane potential was indicated by the ratio of the fluorescence density before and after the application of ATP. The ratio increase represents the membrane potential depolarization. The HN4 cells were transfected with CLIC4 siRNA (CLIC4 siRNA) or scrambled siRNA (Con siRNA). Values are shown as the mean $\pm$ SEM. n = 5. *P < 0.05 vs. control (Con siRNA) group. [Please click here to view a larger version of this figure.](#)

Discussion

It is well documented that Cl⁻ channels are essential in maintaining the hemostasis of the internal environment and play important roles in cell proliferation and apoptosis^{15,16}. Therefore, understanding the relationship between ion channel-targeted intervention and apoptosis is of great need and significance to find a better therapeutic approach for various cancers¹⁷. Mitochondria maintain the normal biological state of a cell and its function highly relates to its membrane permeability and transmembrane potential. Apart from cancer, accumulating neuropathological investigations have documented that mitochondrial abnormalities contribute to myopathy, cardiovascular disease, and neurological symptoms including sensorineural deafness, cerebellar ataxia, dementia, and epilepsy^{18,19}. All these have spurred the research in mitochondrial-targeted intervention with which to ameliorate clinical treatment. Rh123, a mitochondrion-targeted fluorescence dye, can penetrate the cell membrane, and serves as a universal fluorescence probe to detect MMP. In the early state of cell apoptosis, opening of MPTP elevates the mitochondrial membrane permeability allowing Rh123 to be released outside the mitochondria and finally stronger green fluorescence signal may be detected. Under this situation, bilateral ions distribute freely and lead to the rapid drop of MMP causing decoupling of the electron transport chain and decreased ATP production, which severely affects the normal energy supply of cells. Additionally, opening of MPTP triggers the outflow of pro-apoptotic bioactive compounds including Cyt C, apoptosis inducing factor, apoptotic protease activating factor-1, and ROS, and finally leads to irreversible apoptosis^{20,21}.

ATP may induce mitochondrial membrane depolarization as demonstrated by elevated fluorescence of Rh123. By comparing ATP-induced mitochondrial membrane depolarization of cells under different treatments, it is possible to decipher the biological function of the change in mitochondria and the degree and state of apoptosis²². Both common fluorescence microscope and confocal laser scanning fluorescence microscope can achieve real-time monitoring of MMP via recording the fluctuation of fluorescence intensity of Rh123, but advantages and shortcomings of both methods need to be considered. Compared with the common fluorescence microscope, the images captured by confocal laser scanning fluorescence microscope have higher resolution and quality. Therefore, more subcellular details can be visualized. Additionally, it diminishes the impacts of the light cross-talk when multiple fluorescence dyes are used. Hence, the confocal microscope can precisely record the real-time fluorescence fluctuation of Rh123 and reflect the real cellular bioactivity. However, our methods to monitor MMP need continuously recording for a relative long time and a series of cytotoxins, including single oxygen and ROS, may be produced under the laser radiation leading to cell damage²³. Moreover, high laser intensity usually causes fading of fluorescence dyes during the continuous scanning²⁴. By contrast, the common fluorescence microscope cannot take images with high quality like the confocal microscope, but it does not have

the aforementioned adverse effects and thus makes long time recording possible. By comparing our data from both common fluorescence microscope and confocal microscope, no obvious difference could be found, which indicates that the common fluorescence microscope is competent enough for monitoring the fluctuation of MMP, as well as cost-effective and more convenient data analysis. HN4 cells were seeded on coverslips before the experiment. Although polylysine is applied to enhance the cellular attachment, a small fraction of cells still detached from the coverslips and rough operation leads to the impairment of cell viability. Besides, adding Rh123 or ATP into the chamber, care should be taken to not touch the chamber and coverslips.

Besides Rh123, DioC6, JC-1, and tetramethyl rhodamine methyl ester (TMRM) are fluorescence dyes that may be used to detect the MMP. Instead of real-time recording via microscope, flow cytometry also can accomplish this task. However, recording the real-time change of fluorescence intensity with the microscope is more suitable to discover the cellular bioactivity and generate intuitionistic images, giving more reliable results.

Disclosures

The authors have nothing to disclose.

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References

- Chen, J., Liao, W., Gao, W., Huang, J., & Gao, Y. Intermittent hypoxia protects cerebral mitochondrial function from calcium overload. *Acta Neurol Belg.* **113**, 507-513 (2013).
- Gogol, P., Trzcińska, M., & Bryła, M. Motility, mitochondrial membrane potential and ATP content of rabbit spermatozoa stored in extender supplemented with GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide. *Pol J Vet Sci.* **17** (2014).
- Machiels, J. P. *et al.* Advances in the management of squamous cell carcinoma of the head and neck. *F1000Prime Rep.* **6**, 44 (2014).
- Behera, M. *et al.* Concurrent therapy with taxane versus non-taxane containing regimens in locally advanced squamous cell carcinomas of the head and neck (SCCHN): a systematic review. *Oral Oncol.* **50**, 888-894 (2014).
- Pancari, P., & Mehra, R. Systemic therapy for squamous cell carcinoma of the head and neck. *Surgical Oncology Clinics of North America.* **24**, 437-454 (2015).
- Peretti, M. *et al.* Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. *Biochim Biophys Acta.* **1848**, 2523-2531 (2015).
- Jentsch, T. J., Stein, V., Weinreich, F., & Zdebik, A. A. Molecular structure and physiological function of chloride channels. *Physiol Rev.* **82**, 503-568 (2002).
- Ponnalagu, D. *et al.* Data supporting characterization of CLIC1, CLIC4, CLIC5 and DmCLIC antibodies and localization of CLICs in endoplasmic reticulum of cardiomyocytes. *Data Brief.* **7**, 1038-1044 (2016).
- Fernandez-Salas, E. *et al.* mtCLIC/CLIC4, an Organellar Chloride Channel Protein, Is Increased by DNA Damage and Participates in the Apoptotic Response to p53. *Mol Cell Biol.* **22**, 3610-3620 (2002).
- Suh, K. S., Mutoh, M., Gerdes, M., & Yuspa, S. H. CLIC4, an intracellular chloride channel protein, is a novel molecular target for cancer therapy. *J Invest Derm Symp P.* **10**, 105-109 (2005).
- Zhong, J., Kong, X., Zhang, H., Yu, C., Xu, Y., Kang, J., Yu, H., Yi, H., Yang, X., & Sun, L. Inhibition of CLIC4 Enhances Autophagy and Triggers Mitochondrial and ER Stress-Induced Apoptosis in Human Glioma U251 Cells under Starvation. *PLoS one.* **7**, e39378 (2012).
- Ponnalagu, D., & Singh, H. Anion Channels of Mitochondria. *Handbook of Experimental Pharmacology.* (2016).
- Leanza, L. *et al.* Intracellular ion channels and cancer. *Front Physiol.* **4**, 227 (2013).
- Kim, S. Y., Chu, K. C., Lee, H. R., Lee, K. S., & Carey, T. E. Establishment and characterization of nine new head and neck cancer cell lines. *Acta Oto-Laryngologica.* **117**, 775-784 (1997).
- Jia, L., Liu, W., Guan, L., Lu, M., & Wang, K. Inhibition of Calcium-Activated Chloride Channel ANO1/TMEM16A Suppresses Tumor Growth and Invasion in Human Lung Cancer. *PLoS one.* **10**, e0136584 (2015).
- Xu, Y. *et al.* Suppression of CLIC4/mtCLIC enhances hydrogen peroxide-induced apoptosis in C6 glioma cells. *Oncol Rep.* **29**, 1483-1491 (2013).
- Zhu, W., Wang, X., Zhou, Y., & Wang, H. C2-ceramide induces cell death and protective autophagy in head and neck squamous cell carcinoma cells. *Int J Mol Sci.* **15**, 3336-3355 (2014).
- McFarland, R., Taylor, R. W., & Turnbull, D. M. A neurological perspective on mitochondrial disease. *Lancet Neurol.* **9**, 829-840 (2010).
- Alston, C. L., Rocha, M. C., Lax, N. Z., Turnbull, D. M., & Taylor, R. W. The genetics and pathology of mitochondrial disease. *J Pathol.* **241**, 236-250 (2017).
- Zhang, W., Wang, X., & Chen, T. Resveratrol induces mitochondria-mediated AIF and to a lesser extent caspase-9-dependent apoptosis in human lung adenocarcinoma ASTC-a-1 cells. *Mol Cell Biochem.* **354**, 29-37 (2011).
- Bernardi, P., & Rasola, A. Calcium and cell death: the mitochondrial connection. *Subcell Biochem.* **45**, 481-506 (2007).
- Perveen, S., Yang, J. S., Ha, T. J., & Yoon, S. H. Cyanidin-3-glucoside Inhibits ATP-induced Intracellular Free Ca²⁺ Concentration, ROS Formation and Mitochondrial Depolarization in PC12 Cells. *Korean J Physiol Pharmacol.* **18**, 297-305 (2014).
- Paddock, S. W. Principles and practices of laser scanning confocal microscopy. *Mol Biotechnol.* **16**, 127-149 (2000).
- Paddock, S. W. To boldly glow... applications of laser scanning confocal microscopy in developmental biology. *BioEssays : news and reviews in molecular, cellular and developmental biology.* **16**, 357-365 (1994).