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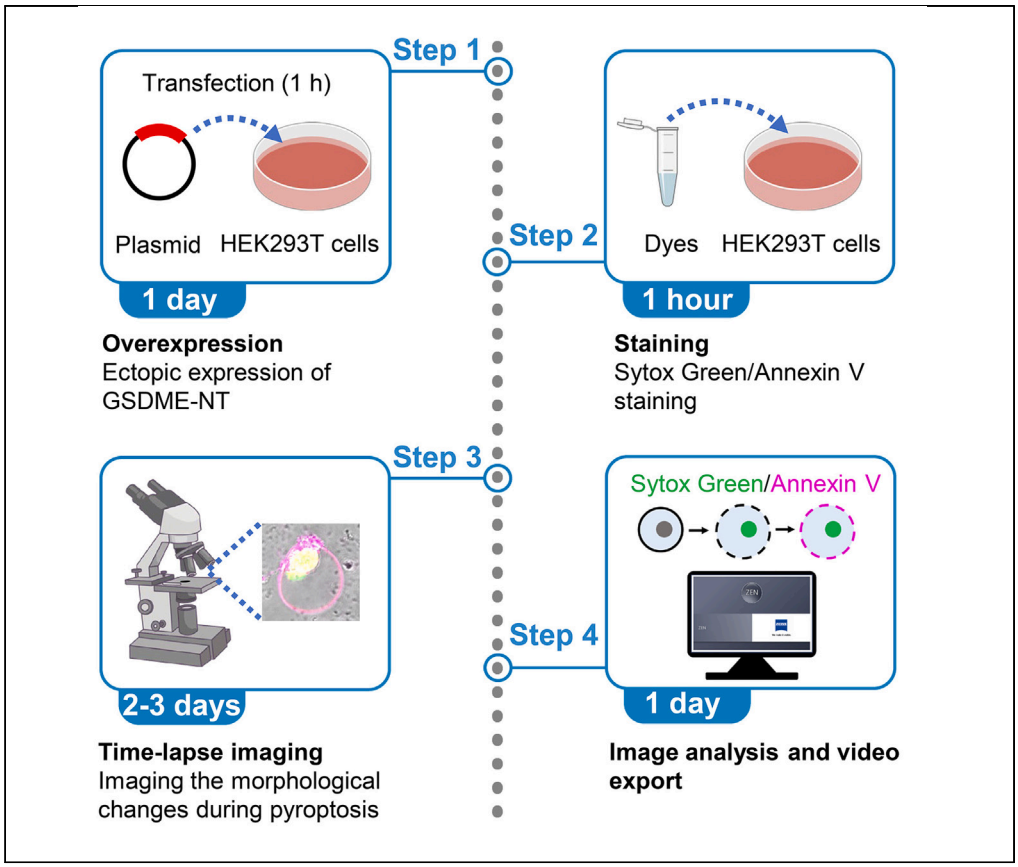
Protocol

Time-lapse live-cell imaging of pyroptosis by confocal microscopy



Pyroptosis is a type of regulated necrosis executed by gasdermin. Osmotic cell swelling and membrane perforation are the key features of pyroptosis. This protocol presents time-lapse imaging of morphological changes during pyroptosis using a confocal microscope. We describe the step-by-step ectopic expression of gasdermin, cell staining with nuclear and membrane probes, and visualization of pyroptosis by time-lapse imaging. This protocol is applicable to monitoring pyroptosis in various situations.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Shuai Jiang,
Kunpeng Qin, Li Sun

sjiang@qdio.ac.cn (S.J.)
lsun@qdio.ac.cn (L.S.)

Highlights

Time-lapse imaging illustrates cell osmotic swelling during pyroptosis

Cell nuclear and membrane staining demonstrate membrane perforation during pyroptosis

This protocol is applicable to imaging pyroptosis under different situations

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Time-lapse live-cell imaging of pyroptosis by confocal microscopy

Shuai Jiang,[1,2,3,4,*] Kunpeng Qin,[1,2,3] and Li Sun[1,2,3,5,*]

[1]CAS and Shandong Province Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China

[2]Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, China

[3]College of Earth and Planetary Sciences, University of Chinese Academy of Sciences, Beijing, China

[4]Technical contact

[5]Lead contact

*Correspondence: sjiang@qdio.ac.cn (S.J.), lsun@qdio.ac.cn (L.S.)
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SUMMARY

Pyroptosis is a type of regulated necrosis executed by gasdermin. Osmotic cell swelling and membrane perforation are the key features of pyroptosis. This protocol presents time-lapse imaging of morphological changes during pyroptosis using a confocal microscope. We describe the step-by-step ectopic expression of gasdermin, cell staining with nuclear and membrane probes, and visualization of pyroptosis by time-lapse imaging. This protocol is applicable to monitoring pyroptosis in various situations.

For complete details on the use and execution of this protocol, please refer to Qin et al. (2023).[1]

BEFORE YOU BEGIN

Pyroptosis is a type of programmed necrosis executed by gasdermin (GSDM) family members.[2] During pyroptosis, gasdermin is cleaved by protease and releases the pore-forming N-terminal (NT) fragment, which perforates the cell membrane and induces osmotic lysis. For the inflammasome-mediated pyroptosis, the canonical and non-canonical inflammasome pathways activate caspase-1/4/5/11, which cleave gasdermin D (GSDMD) to produce the pyroptosis-inducing NT fragment.[3–5] Different from GSDMD, gasdermin E (GSDME) is cleaved by caspase-3 and granzyme B to release the NT fragment that initiates pyroptosis.[6–8] In this protocol, we use a GSDME from Pacific abalone *Haliotis discus* for the induction of pyroptosis,[1] and describe step-by-step GSDME ectopic expression, cell staining and time-lapse imaging of pyroptosis by confocal microscopy. Before starting the experiment, the GSDME NT (GSDME-NT) domain, which possesses pyroptosis-inducing activity, is cloned into a mammalian expression vector pmCherry-N1. In this protocol, the immortalized human embryonic kidney (HEK293T) cells are used for the ectopic expression of GSDME-NT. Besides HEK293T cells, we have also used HeLa cells for ectopic expression and time-lapse imaging of pyroptosis. Since time-lapse imaging of pyroptosis usually lasts 2–3 days, the researchers should have a confocal microscope equipped with an environmental control chamber to maintain 37°C and humidified 5% CO_2 to support cell growth. We also recommend to prepare the commercially available nuclear dyes (e.g., Sytox Green) and inner membrane fluorescent probes (e.g., Annexin V), which are often used to observe membrane perforation and osmotic swelling during pyroptosis. This protocol is also useful to examine pyroptosis in other situations, such as infection, cancer and inflammatory diseases.

Preparation of cells

Timing: 5–7 days

1. Thaw the frozen HEK293T cells rapidly in a 37°C water bath. After centrifugation at 500 *g* at 24°C for 5 min, discard the freezing medium and re-suspend the cells with pre-warmed Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.
2. Culture the cells in a T-25 flask at 37°C in a 5% CO_2 incubator. Grow the cells to 80%–90% confluence, and culture the cells at least two passages to reach the exponential growing phase.

Endotoxin-free plasmid DNA purification

Timing: 2 days

3. Grow the *Escherichia coli* (*E. coli*) Trelief 5α cells that harbor the recombinant pmCherry-N1-GSDME-NT expression vector in Luria-Bertani broth supplemented with 50 μg/mL of kanamycin and shaken at 220 rpm at 37°C for 12–17 h.
4. Pellet the *E. coli* cells by centrifugation at 3000 *g* at 24°C for 15 min.
5. Obtain the endotoxin-free plasmid DNA (endotoxin < 0.1 EU/μg plasmid DNA) by using Endo-Free Plasmid DNA Kit (OMEGA) according to manufacturer's instruction (http://www.omegabiotek.com.cn/template/productShow.aspx?m=129002&i=100000012774922).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli Trelief 5α	Tsingke Biological Technology	Cat.#TSC01
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium	Corning	Cat.#10-013-CVRC
Fetal bovine serum	Sigma-Aldrich	Cat.#F8687
Trypsin-EDTA	Sigma-Aldrich	Cat.#SM-2003
PolyJet transfection reagent	SignaGen Laboratories	Cat.#L100688
Annexin V Alexa Fluor 647	Thermo Fisher Scientific	Cat.#R37175
Sytox Green	Thermo Fisher Scientific	Cat.#S7020
$CaCl_2$	Sigma-Aldrich	Cat.#C5670
Critical commercial assays		
Endo-Free plasmid DNA kit	OMEGA	Cat.#D6950-02
Experimental models: Cell lines		
HEK293T	ATCC	Cat.#CRL-3216
Recombinant DNA		
pmCherry-N1-GSDME-NT	This paper	N/A
Software and algorithms		
Zen	Zeiss	http://www.zeiss.com
Other		
LSM710 confocal microscope system equipped with environmental control chamber	Zeiss	http://www.zeiss.com
Steri cycle CO_2 incubator	Thermo Fisher Scientific	https://www.thermofisher.com/
NanoPhotometer N60	Implen Spectrophotometer	https://www.implen.de/
Eppendorf 5424	Eppendorf	https://www.eppendorf.com
35-mm glass-bottom dish	Corning	Cat.#801002
T-25 flask	Corning	Cat.#3289

STEP-BY-STEP METHOD DETAILS

Ectopic expression of GSDME-NT

Timing: 1 day

1. Digest the exponentially growing HEK293T cells by trypsin-EDTA to single cells. Plate the cells (approximately 5 $\times 10^5$ cells) in 35-mm glass-bottomed culture dishes for 12–17 h to reach 60%–70% confluency before transfection.
2. Discard the medium, and add fresh DMEM (supplemented with 10% FBS) to the cells at 30 min before transfection.
3. Prepare the transfection complex by using PolyJet transfection reagent.
   a. Gently dilute the plasmid DNA (1 μg) and PolyJet transfection reagent (3 μL) with 50 μL serum-free DMEM.
   b. Add the diluted transfection reagent to the diluted plasmid DNA and mix gently.
   c. Incubate the mixture for 15 min to prepare the transfection complex.
4. Add the transfection complex drop-wise to the cells and gently swirl the cell culture dishes.
5. After transfection for 24 h, check the expression level of GSDME-NT using a fluorescence microscope.

Alternatives: Some other commercially available transfection reagents, e.g., lipofectamine 3000 transfection reagent (Thermo Fisher Scientific), can also be used for cell transfection. In addition, HeLa cells can also be used for the ectopic expression of GSDME-NT.

⚠ CRITICAL: To prepare the transfection complex for the negative control transfection, add the diluted transfection reagent to serum-free DMEM and incubate the mixture as described in step 3.

⚠ CRITICAL: Because HEK293T cells are semi-adhesive, all steps, including medium replacement, transfection, cell staining, and microscopic observation, should be careful to avoid cell detachment or damage.

Staining of cell nucleus and membrane

Timing: 1 h

6. Dilute Sytox Green and Annexin V-Alexa Fluor 647 in the cell culture medium to the final concentration of 1 μM (Sytox Green) or add one drop Annexin V-Alexa Fluor 647 solution per 10^5 cells according to the manufacturer's instruction (https://www.thermofisher.com/order/catalog/product/R37175).
7. Incubate the cells with the Sytox Green and Annexin V-Alexa Fluor 647 at 37°C in dark for 15 min.

⚠ CRITICAL: Due to the calcium-dependent binding to phosphatidylserine, add sterile $CaCl_2$ stock solution to the cell culture medium to reach a final concentration of 2.5 mM before Annexin V staining.

Time-lapse imaging of pyroptosis

Timing: 3–4 days

8. Start the LSM710 confocal microscope, and pre-warm the laser source.
9. Equip the microscope with an environmental control chamber. Warm up the environment chamber to 37°C, and maintain a supply of humidified 5% CO_2.
10. Place the cell culture dish into the environmental control chamber.
11. Choose the desired objective for cell imaging. Typically, 40 × (NA1.2) magnification objective is preferred.
12. Locate a view field of interest and then go to the software operation panel. Choose the fluorescence channels and pseudo-colors that match the dyes used in the experiment. To view the brightfield image, select the channel corresponding to brightfield.

13. Adjust the z-axis to get a clear view of interest. Set the excitation laser power, and optimize the gain of fluorescence to avoid low fluorescent intensity or overexposure. The "Pinhole" for each fluorescent channel is set at 1 Airy Unit (AU). Adjust the gain and offset of each fluorescent channel to as required before imaging.
14. Take 512 × 512 time-lapse images at 3–5 min intervals for a total of 7 h or longer.
15. Save the time series images, and represent the images as "Contents of Image window-series" or "Full resolution image window-series". Adjust frames per second and export the time-lapse imaging video. The expected outcome could be the Method video S1.

Alternatives: The confocal microscope from Leica and Nikon can be used as an alternative to that of Zeiss.

⚠ CRITICAL: Warm up the environmental control chamber and supply humidified 5% CO_2 at least 1 h before imaging.

⚠ CRITICAL: The concentrations of the fluorescent dyes/probes and the gain and offset of each fluorescent channel should be adjusted by using positive and negative control before time-lapse imaging.

⚠ CRITICAL: Repeat time-lapse imaging (steps 11–15) to generate a high-quality video.

EXPECTED OUTCOMES

Pyroptosis is an inflammatory type of regulated necrotic cell death.[9] GSDM executes pyroptosis via its NT pore-forming domain.[3–5] Cell swelling and perforation are the hall-markers of pyroptosis induced by GSDM-NT.[10] In this protocol, we transfect HEK293T cells with the vector expressing abalone GSDME-NT, which possesses robust pyroptosis-inducing activity.[1] Compared with the negative control, abalone GSDME-NT is expressed in cells and could translocate to the cytoplasmic membrane (Figures 1 and S1), and induces rapid cell membrane perforation and osmotic swelling (Figure 2, Method video S1), characterized by Sytox Green uptake and Annexin V staining (Figures 1 and 2). This protocol can be used to determine the pyroptosis-inducing activity of GSDM fragments or GSDM homologs, and can also be adapted for imaging pyroptosis under pathological conditions.

LIMITATIONS

This step-by-step protocol for time-lapse imaging of pyroptosis is straightforward to visualize rapid membrane swelling and perforation, which are typical morphological features of pyroptotic cell death. Although this protocol is suitable for imaging pyroptosis under different conditions, several issues need to be considered.

First, the time-lapse imaging is time consuming and usually takes 3–4 days to acquire high-qualified images. For the canonical inflammasome activated pyroptosis, detecting the inflammasome (e.g., NLRP3) and ASC speck assembly is a faster way to study the early stage of pyroptosis.[11] Second, an environmental control chamber with accurate control of heat and CO_2 is required to support the long-term survival of cells, and the chamber should be germ-free during imaging. Third, the concentration of the fluorescent dye/probe and the gain and offset of each fluorescent channel should be adjusted by using positive and negative controls before imaging. Fourth, the release of lactate dehydrogenase and generation of pore-forming GSDM-NT are other key markers of pyroptosis, and can serve as complementary evidences to the occurrence of pyroptosis.

TROUBLESHOOTING

Problem 1

The cultured cells are contaminated with microbes.

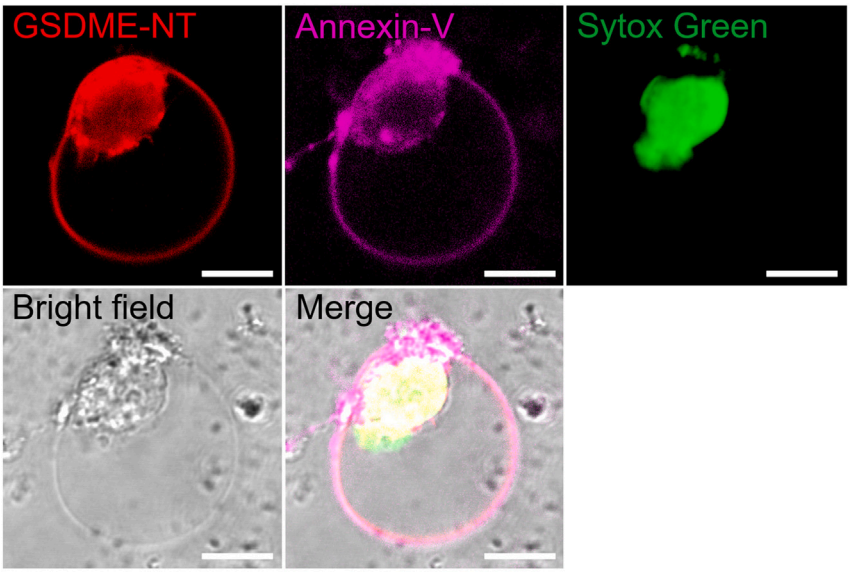

Figure 1. Morphological characteristics of a pyroptotic cell expressing abalone GSDME-NT
HEK293T was transfected with the vector expressing mCherry tagged abalone GSDME-NT for 24 h. The cells were then stained with Sytox Green and Alexa Fluor 647-Annexin V. Images were captured with a confocal microscope. Scale bar: 10 μm.

Potential solution

- Always wear clean gloves and lab coat, and use sterile labware during the experiment (all steps mentioned in this protocol except for image/video processing).
- Clean the environmental control chamber of the confocal microscope with 70% ethanol or other disinfectants before imaging (step 9).
- Minimize the exposure of cells placed in non-sterile conditions (steps 6, 7 and 10).
- The fluorescent probes or reagents should be filtered through 0.22 μm sterile filters to remove bacteria and fungi (steps 6 and 7).

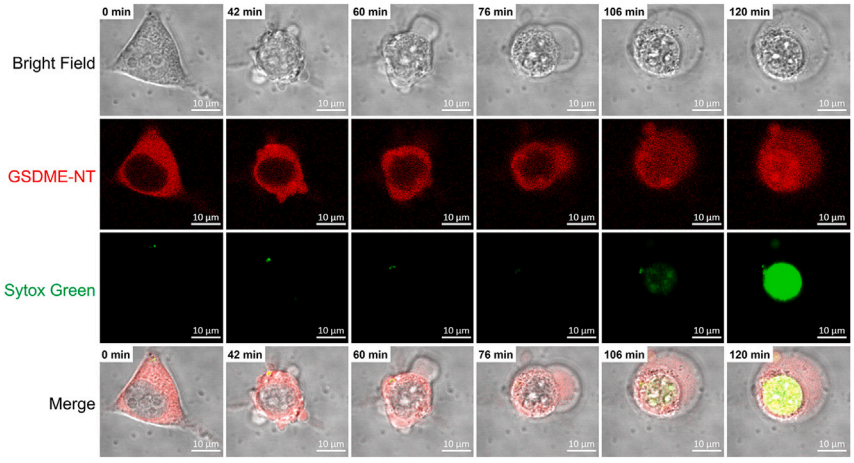

Figure 2. Time-lapse imaging of a cell expressing abalone GSDME-NT
mCherry tagged GSDME-NT was overexpressed in HEK293T cells. After transfection for 24 h, the cells were incubated with Sytox Green, and the time-lapse images of pyroptosis were captured with a confocal microscope. Scale bar: 10 μm.

- Add antibiotics, such as penicillin and streptomycin, to the cell culture to prevent bacterial contamination. Anti-mycoplasma reagents, for example plasmocin (Invivogen), can also be used to prevent mycoplasma contamination (step 2).

Problem 2

The dead cells fluctuate and cause focus drift during time-lapse imaging.

Potential solution

Adjust the z-axis to re-focus on the pyroptotic cells during imaging. Autofocus devices, such as Definite Focus (Carl Zeiss), are also recommended to maintain stable focus during imaging (step 14).

Problem 3

The fluorescence is quenched during imaging.

Potential solution

Use stable fluorescent protein tags, such as enhanced green fluorescent protein (EGFP) and mCherry, to the target protein during time-lapse imaging. For cell nucleus or membrane staining, photostable reagents or conjugates should be used (steps 6, 7).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Li Sun (lsun@qdio.ac.cn).

Materials availability

Plasmids generated in this study will be made available on request to the lead contact. This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102708.

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AUTHOR CONTRIBUTIONS

S.J. and L.S. conceived the idea, designed the study, and obtained the funding. S.J. and K.Q. conducted the experiments and analyzed the data. S.J. wrote the first draft of the manuscript. L.S. revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

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Supplemental Materials

Figure S1. Ectopic expression of abalone GSDME-NT in HEK293T cells. GSDME-NT is tagged with mCherry and indicated by red color. Scale bar: 50 μm. Related to step 5.