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Title: Live-Cell Imaging of Lysosomal Membrane Permeabilization During Necroptosis

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES, all done**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: 12/08/2025

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 14
Number of Shots: 26

Introduction

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Katia Andrade:** This protocol outlines a live-cell imaging method to monitor lysosomal membrane permeabilization during necroptosis using fluorescent probes detecting integrity and acidification changes.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

NOTE: Use IMG_1445.mov

~~What are the most recent developments in your field of research?~~

1.2. **Katia Andrade:** Recent advances include real-time imaging approaches revealing dynamic organelles changes during regulated cell death pathways.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What are the current experimental challenges?~~

1.3. **Katia Andrade:** **NOTE: This statement was adding post-filming. Use IMG_1446.mov**

CONCLUSION:

~~What significant findings have you established in your field?~~

1.4. **Katia Andrade:** We demonstrated that lysosomal membrane permeabilization can be directly visualized in living cell undergoing necroptosis induction.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **NOTE: Use IMG_1449.mov**

~~What research gap are you addressing with your protocol?~~

1.5. **Katia Andrade:** This protocol shows that combining LysoTracker with fluorescein-labeled dextran beads allows real-time monitoring of lysosomal pH and cargo release dynamics.

INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **NOTE: Use IMG_1450.mov**

~~What advantage does your protocol offer compared to other techniques?~~

1.6. ~~**Katia Andrade:** My protocol provides a unique, dual readout on both lysosomal functionality and structural integrity in live cells.~~
NOTE: This statement not filmed.

~~How will your findings advance research in your field?~~

1.7. **Katia Andrade:** **NOTE: This statement was adding post-filming. Use IMG_1456.mov**

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

NOTE: Protocol scripted from Footage

2. Live-Cell Confocal Imaging for Monitoring Lysosomal Membrane Permeabilization

Demonstrator: Katia Andrade

2.1. To begin, plate 5,000 cells in 2 milliliters of antibiotic supplemented DMEM media into a sterile glass-bottom dish **[1-TXT]**. Place the dish in an incubator set to 37 degrees Celsius with 5 percent carbon dioxide and incubate the cells overnight **[2]**.

2.1.1. LAB MEDIA: GX011140 01:27-01:45
TXT: Media supplementation: 10% FBS, 1% Penicillin and Streptomycin

2.1.2. LAB MEDIA: GX011141 00:12 – 00:22
AND GX011142 00:00 – 00:04

2.2. Add 20 microliters of dextran green beads to 25 micrograms per milliliter in fresh antibiotic-supplemented DMEM media to dilute it **[1]**.

2.2.1. LAB MEDIA: GX011145 00:21 – 01:04

2.3. Remove the old medium from the dish **[1]** and replace it with 2 milliliters of the bead-containing supplemented DMEM media **[2]**. Place the dish in an incubator set to 37 degrees Celsius with 5 percent carbon dioxide and incubate for 24 hours to allow internalization of beads into lysosomes **[3]**.

2.3.1. LAB MEDIA: GX011147 00:05 – 00:10

2.3.2. LAB MEDIA: GX011147 00:19 – 00:30

2.3.3. LAB MEDIA: GX011147 00:46 – 00:50
AND GX011148 00:00 - 00:05

2.4. Now, add 2 microliters of 1 millimolar Lysotracker stock solution to 2 milliliters of fresh DMEM media **[1-TXT]**. Replace the old medium with this staining solution **[2]**.

2.4.1. LAB MEDIA: GX011150 00:00 – 00:16
TXT: Final concentration: 1 µM

2.4.2. LAB MEDIA: GX011151 00:05-00:09, 00:19 – 00:32

2.5. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 2 hours **[1]**.

2.5.1. LAB MEDIA: GX011152 00:05 – 00:10

2.6. Turn on the confocal system components by switching on the personal computer,

microscope, scanner power, laser power, laser emission, and Leica power **[1]**.

2.6.1. LAB MEDIA: GX011153 00:00 – 00:10

2.7. Turn on the environmental controls including the temperature controller called The Cube and the carbon dioxide gas mixer controller called The Brick **[1]**. Open the carbon dioxide and oxygen supply **[2]**.

2.7.1. LAB MEDIA: GX011153 00:11 – 00:16

2.7.2. LAB MEDIA: GX011154 00:00 – 00:10

2.8. Set the temperature controller to 37 degrees Celsius **[1]**. Set the gas and humidity controller to 5 percent carbon dioxide and 95 percent humidity **[2]**.

2.8.1. LAB MEDIA: GX011155 00:00 – 00:03

2.8.2. LAB MEDIA: GX011155 00:17 – 00:33
AND
GX011184 00:04-00:06

2.9. Assemble the imaging chamber **[1-TXT]**.

2.9.1. LAB MEDIA: GX011157 00:30 – 00:35
AND
TEXT ON PLAIN BACKGROUND:
Use 63X or 1.4 NA oil immersion for confocal imaging
Excitation : 488 nm (green), 555 nm (red)
Detector: Hybrid (488 nm), PMT (555nm)

2.10. After staining, wash the cells three times with 2 milliliters of PBS **[1]**. Then add 2 milliliters of fresh supplemented DMEM media **[2]**. Apply immersion oil onto the objective lens before imaging **[3]**. Mount the dish on the microscope stage **[4]**.

2.10.1. LAB MEDIA: GX011170 00:49 – 00:56, 01:13-01:15, 01:21-01:25

2.10.2. LAB MEDIA: GX011170 02:50 – 03:10

2.10.3. LAB MEDIA: GX011163 00:00 – 00:05

2.10.4. LAB MEDIA: GX011165 00:09 – 00:20

2.11. Adjust the laser intensity up to 3 percent, but not more than 5 percent **[1]**. Then adjust the detector gain in the microscope software to optimize the signal **[2]**. Minimize the background by adjusting the contrast bar **[3]**.

2.11.1. LAB MEDIA**:** LAS X 12_3_2025 10_31_39 AM 00:00 – 00:08

2.11.2. LAB MEDIA**:** LAS X 12_3_2025 1_54_21 PM 00:04 – 00:15

2.11.3. LAB MEDIA: LAS-X-12_3_2025-10_59_23-AM 00:00 – 00:12

2.12. Acquire baseline images of untreated cells, which serve as the control condition **[1]**.

2.12.1. LAB MEDIA**:** LAS X 12_3_2025 11_02_12 AM **Timestamps**: 01:19 – 01:25

2.13. Return the plate to the laminar flow hood and add 1 milliliter antibiotic-DMEM supplemented with growth factors **[1-TXT]**.

2.13.1. LAB MEDIA: GX011183 00:54 – 01:12

TXT: Media supplementation: 100 ng/mL of TNF-α (T), 500 nM of SMAC mimetic (S), 100 µM of Z-VAD-FMK (Z)

2.14. In the acquisition software, set the **mode** to **xyt**, **format** to **512 x 512** *(Five-twelve-by-Five-twelve)*, **speed** to **400**, **zoom** to **3**, **line average** to **1**, **line accumulation** to **3**, **frame accumulation** to **1**, **time interval** to **3 min** *(minutes)*, **duration** to **8h** *(hours)*, and enable **autofocus [1]**. Click **Start** to begin the time-lapse acquisition **[2]**.

2.14.1. LAB MEDIA**:** LAS X 12_3_2025 11_02_12 AM 00:00 – 01:10:

2.14.2. LAB MEDIA**:** LAS X 12_3_2025 11_02_12 AM 01:30 – 01:34

Results

3. Results

3.1. Initial imaging confirmed the presence of stable puncta of LysoTracker **[1]** and fluorescein-labeled dextran, indicating intact lysosomes before treatment **[2]**.

3.1.1. LAB MEDIA: Figure 3. *Video editor: Highlight the red fluorescent spots labeled "LysoTracker" in the "Before" column.*

3.1.2. LAB MEDIA: Figure 3. *Video editor: Highlight the green fluorescent spots labeled "Dextran" in the "Before" column.*

3.2. Four hours after treatment with T/S/Z *(T-S-Z)* , LysoTracker fluorescence was noticeably reduced **[1-TXT]**, while dextran began to show diffuse cytosolic distribution, indicating initial lysosomal membrane permeabilization **[2]**.

3.2.1. LAB MEDIA: Figure 3.
TXT: T: Tumor Necrosis Factor Alpha;
S: SMAC mimetic;
Z: Z-VAD-FMK
Video editor: Highlight the visibly dimmer red fluorescence in the "4 hr after" column under "LysoTracker".

3.2.2. LAB MEDIA: Figure 3. Video editor: Highlight the partial spread of green fluorescence in the "4 hr after" column under "Dextran".

3.3. By 8 hours after treatment, LysoTracker signal had almost completely disappeared **[1]**, while many cells retained green dextran puncta with diffuse green cytosolic signal **[2]**.

3.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the absence of red signal in the "8 hr after" column under "LysoTracker".*

3.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the green puncta and surrounding diffuse green fluorescence in the "8 hr after" column under "Dextran".*

Pronunciation Guide:

Lysosomal
Pronunciation link: https://www.merriam-webster.com/dictionary/lysosomal
IPA: No confirmed IPA found
Phonetic Spelling: ly·suh·SOH·muhl
Lysosome
Pronunciation link: https://en.wiktionary.org/wiki/lysosome
IPA: /ˈlaɪsəˌsoʊm/
Phonetic Spelling: LY·suh·sohm
Permeabilization
Pronunciation link: No confirmed link found
IPA: No confirmed IPA found
Phonetic Spelling: pur·mee·uh·buh·luh·ZAY·shuhn
Necroptosis
Pronunciation link: https://www.howtopronounce.com/necroptosis
IPA: No confirmed IPA found
Phonetic Spelling: neh·KRAH(p)·TOH·suhs
Confocal
Pronunciation link: https://www.merriam-webster.com/dictionary/confocal
IPA: /kənˈfoʊkəl/
Phonetic Spelling: kuhn·FOH·kuhl
Fluorescein
Pronunciation link: https://www.howtopronounce.com/fluorescein
IPA: No confirmed IPA found
Phonetic Spelling: floor·EH·seen
Dextran
Pronunciation link: No confirmed link found
IPA: No confirmed IPA found
Phonetic Spelling: DEKS·tran
LysoTracker
Pronunciation link: No confirmed link found
IPA: No confirmed IPA found
Phonetic Spelling: LY·zoh·TRAK·er
Internalization
Pronunciation link: https://www.merriam-webster.com/dictionary/internalization
IPA: No confirmed IPA found
Phonetic Spelling: in·tur·nuhl·uh·ZAY·shuhn
Acidification
Pronunciation link: https://www.merriam-webster.com/dictionary/acidification
IPA: No confirmed IPA found
Phonetic Spelling: uh·sid·uh·fih·KAY·shuhn

- **Organelles**
Pronunciation link: https://www.merriam-webster.com/dictionary/organelle
IPA: No confirmed IPA found
Phonetic Spelling: or·guh·NELS
- **Antibiotic**
Pronunciation link: https://www.merriam-webster.com/dictionary/antibiotic
IPA: No confirmed IPA found
Phonetic Spelling: an·tye·bye·AH·tik
- **Supplemented**
Pronunciation link: https://www.merriam-webster.com/dictionary/supplemented
IPA: No confirmed IPA found
Phonetic Spelling: SUP·luh·men·tid
- **Incubator**
Pronunciation link: https://www.merriam-webster.com/dictionary/incubator
IPA: No confirmed IPA found
Phonetic Spelling: IN·kyuh·bay·ter
- **Confocal Imaging**
Pronunciation link: No confirmed link found
IPA: No confirmed IPA found
Phonetic Spelling: kuhn·FOH·kuhl IM·uh·jing
- **Hybrid**
Pronunciation link: https://www.merriam-webster.com/dictionary/hybrid
IPA: No confirmed IPA found
Phonetic Spelling: HY·brid
- **Autofocus**
Pronunciation link: https://www.merriam-webster.com/dictionary/autofocus
IPA: No confirmed IPA found
Phonetic Spelling: AW·toh·FOH·kuhs
- **Time-lapse**
Pronunciation link: https://www.merriam-webster.com/dictionary/time-lapse
IPA: No confirmed IPA found
Phonetic Spelling: TYME·laps
- **Laminar**
Pronunciation link: https://www.merriam-webster.com/dictionary/laminar
IPA: No confirmed IPA found
Phonetic Spelling: LAM·uh·ner
- **Cytosolic**
Pronunciation link: No confirmed link found
IPA: No confirmed IPA found
Phonetic Spelling: SYE·tuh·SAH·lik