Gene Delivery and Analysis of Optogenetic Induction of Lytic Cell Death

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Necroptosis is a form of inflammatory lytic cell death involving active cytokine production and plasma membrane rupture. Progression of necroptosis is tightly regulated in time and space, and its signaling outcomes can shape the local inflammatory environment of cells and tissues. Pharmacological induction of necroptosis is well established, but the diffusive nature of chemical death inducers makes it challenging to study cell-cell communication precisely during necroptosis. Receptor-interacting protein kinase 3, or RIPK3, is a crucial signaling component of necroptosis, acting as a crucial signaling node for both canonical and non-canonical necroptosis. RIPK3 oligomerization is crucial to the formation of the necrosome, which regulates plasma membrane rupture and cytokine production. Commonly used necroptosis inducers can activate multiple downstream signaling pathways, confounding the signaling outcomes of RIPK3-mediated necroptosis. Opsin-free optogenetic techniques may provide an alternative strategy to address this issue. Optogenetics uses light-sensitive protein-protein interaction to modulate cell signaling. Compared to chemical-based approaches, optogenetic strategies allow for spatiotemporal modulation of signal transduction in live cells and animals. We developed an optogenetic system that allows for ligand-free optical control of RIPK3 oligomerization and necroptosis. This article describes the sample preparation, experimental setup, and optimization required to achieve robust optogenetic induction of RIPK3-mediated necroptosis in colorectal HT-29 cells. We expect that this optogenetic system could provide valuable insights into the dynamic nature of lytic cell death. © 2024 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Production of lentivirus encoding the optogenetic RIPK3 system
Support Protocol: Quantification of the titer of lentivirus
Basic Protocol 2: Culturing, chemical transfection, and lentivirus transduction of HT-29 cells
Basic Protocol 3: Optimization of optogenetic stimulation conditions
Basic Protocol 4: Time-stamped live-cell imaging of HT-29 lytic cell death
Basic Protocol 5: Quantification of HT-29 lytic cell death

Keywords: cell signaling • lytic cell death • necroptosis • optogenetic • RIPK3

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INTRODUCTION

Cell death is an essential mechanism to maintain the homeostasis of multicellular organisms. Apoptosis is a form of programmed cell death that clears apoptotic corpses in a "quiet" way, in that minimal inflammatory factors are released. Necrosis, which is typically not programmed, proceeds with membrane rupture and release of cellular contents into the extracellular space, resulting in inflammation. Necroptosis bears features of both apoptosis and necrosis in that cell rupture proceeds with concomitant upregulation of proinflammatory cytokine production. Defective necroptosis has been reported in multiple inflammatory diseases, such as inflammatory bowel diseases (IBD) and neurodegenerative diseases. Therefore, understanding the precise mechanism of necroptosis should benefit the development of future therapeutics.

A well-established canonical necroptosis pathway is initiated by tumor necrosis factor alpha (TNFα). TNFα binding to its receptor, TNF receptor 1 (TNFR1), recruits receptor-interacting protein kinase 1 (RIPK1) and other proteins to form a receptor complex called complex-I at the plasma membrane, which further promotes activation of the nuclear factor kappa B (NF-κB) pathway (Zhu et al., 2018). When apoptosis is blocked by the broad-spectrum caspase inhibitor zVAD-FMK, a membrane-less protein complex called a necrosome is formed, containing RIPK1 and RIPK3 (Cho et al., 2009). RIPK3 phosphorylates mixed lineage kinase domain-like (MLKL) and causes membrane rupture (Dondelinger et al., 2014; Sun et al., 2012). Non-canonical necroptosis also exists, for example, in the case of viral induction, and proceeds through RIPK3-dependent signaling processes. Highly inflammatory, necroptosis is regulated tightly in time and space.

The commonly used pharmacological approaches to stimulate necroptosis may face challenges in delineating the spatial and temporal regulation of necroptosis, in part due to the diffusive nature of chemical death inducers. Additionally, the potential off-target effects of small molecules make it difficult to define the function of specific signaling components such as RIPK3. One alternative strategy to address this issue is optogenetic technology, which engineers cells to harbor light-sensitive signaling molecules. Exposure to visible light induces conformational changes in the light-sensitive signaling molecules and activates downstream signaling pathways. Because light can be controlled precisely in time and space, signaling modulation can be achieved at the single-cell or even subcellular level (Fischer et al., 2022; Liu et al., 2022; Mazraeh & Di Ventura, 2022; Oh et al., 2021).

Here, we demonstrate the optical control of necroptosis in live cells. The system uses cryptochrome protein, a photoactivable protein that can be oligomerized by blue light, to induce RIPK3 activation in live cells. In this article, we introduce the procedure of preparing lentivirus encoding the optogenetic RIPK3 system (Basic Protocol 1 and Support Protocol), lentiviral transduction (Basic Protocol 2), optimization of optogenetic stimulation conditions (Basic Protocol 3), long-term time-stamped live-cell imaging and optogenetic induction (Basic Protocol 4), and data analysis (Basic Protocol 5).

STRATEGIC PLANNING

Successful achievement of optogenetic induction of necroptosis requires high-quality lentivirus, optimized optogenetic stimulation conditions, and familiarity with data analysis algorithms. Several steps can be performed in parallel, for example, Basic Protocols 1 and 3.

CAUTION: All procedures regarding lentivirus production, human cell line culturing, chemical transfection, and lentivirus transduction should be done in a certified biosafety level 2 (BSL2) biosafety cabinet. Please handle all materials with great care throughout the procedures to minimize potential microbe contamination. The imaging room for handling live human cell line imaging should meet the BSL2 requirement as well.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly, including using a certified biosafety class II cabinet (Thermo Fisher 1300 Series A2).

NOTE: All culture incubations are performed in a 37°C, 5% CO_2, 95% air incubator unless otherwise specified.

PRODUCTION OF LENTIVIRUS ENCODING THE OPTOGENETIC RIPK3 SYSTEM

BASIC PROTOCOL 1

This protocol outlines the steps to produce lentivirus encoding the optogenetic RIPK3 system. Lentivirus transduction is essential to achieve effective gene delivery into HT-29 cells (Basic Protocol 2).

Materials

Low-passage (<P15) HEK293T cells (ATCC, cat. no. CRL-3216)
Complete DMEM [supplemented with fetal bovine serum (FBS); see recipe], 37°C
Plasmids:
  Transfer plasmid (for optogenetic RIPK3)
  Packaging plasmid (psPAX2)
  Envelope plasmid (pMD2.G)
TurboFect Transfection Reagent (Thermo Fisher, cat. no. R0532)
Dulbecco's Modified Eagle's Medium (DMEM; Corning, cat. no. 10-013-CV) with antibiotics (1× penicillin/streptomycin, from 10,000 U/ml stock; Corning, cat. no. 30-002-CI) and without FBS
Lenti-X Concentrator (Takara, cat. no. 631231, lot no. 1710388A)
Complete medium of user's choice or Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher, cat. no. 14040141)

100-mm culture plates (Fisherbrand, cat. no. FB012924)
1.5-ml Eppendorf tubes (Thermo Fisher)
15- or 50-ml Falcon tubes (Thermo Fisher)
Standard tabletop centrifuge (Sorvall ST 8 Centrifuge, Thermo Fisher, cat. no. 75007200), room temperature and 4°C
0.4-μm syringe filters, sterile (Milltex PVDF 0.45 μm Sterile Syringe Filter, Millipore/Sigma-Aldrich, cat. no. SLHVR33RS)
10-ml syringes, sterile (Fisherbrand, cat. no. 14-955-459)

Additional reagents and equipment for measuring lentivirus concentration (see Support Protocol)

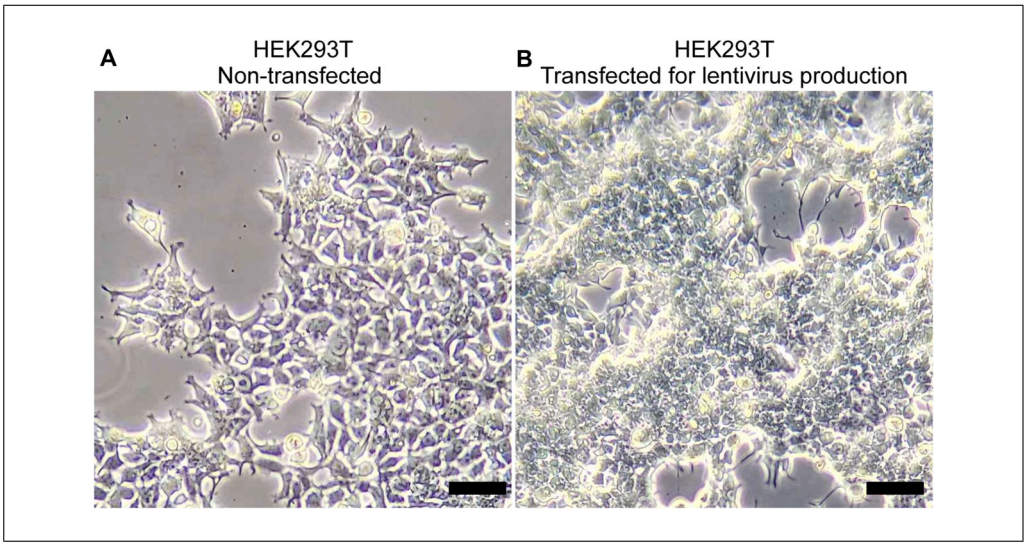

Figure 1 HEK293T cells show unhealthy morphology during lentivirus production. (**A**) Non-transfected HEK293T cells show stretched cell morphology. (**B**) When transfected with the transfer plasmid, psPAX2, and pMD2.G, cells show aggregation and shrinkage, a good sign for potent lentivirus production. Scale bars, 50 µm.

1. Grow low-passage HEK293T cells in a 100-mm culture plate in 10 ml complete DMEM (supplemented with FBS) at a 10% initial seeding density until 85% to 90% confluent to maximize the virus yield.

   Using a 10-ml serological pipet (Thermo Fisher, cat. no. 170356N) and controller (Thermo Fisher S1 Pipet Filler, Thermo Fisher, cat. no. 9501) will make all steps of the protocol much easier to complete.

2. Prepare 800 µl transfection mix in a 1.5-ml Eppendorf tube, with a 3:2:1 molar ratio of transfer/packaging/envelope plasmid and a ratio of DNA/TurboFect Transfection Reagent of 1 µg to 3 µl.

   The weight ratio of transfer (bp)/packaging (bp)/envelope (bp) = 9628:10699:5800 = 1:1.11:0.6. This is the weight ratio to ensure a 1:1:1 molar ratio. Thus, for a molar ratio of 3:2:1, the weight ratio should be 3:2.22:0.6. Distribute 8000 ng total weight using this ratio and measure the weight of each plasmid as 4123 ng (transfer), 3052 ng (packaging), and 825 ng (envelope).

3. Once the transfection mix is made, gently remove the old culture medium and replenish with 7.2 ml DMEM with antibiotics and without FBS (8 ml total volume).

4. Wait for 20 min for the lipid particle to generate complex at room temperature.

5. Add 800 µl of the transfection mix evenly to the plate dropwise.

   Gently add the medium to the side wall of the plate so as not to unexpectedly detach cells.

6. Incubate the transfected cells in a 37°C, 5% CO_2 incubator for 20 hr.

7. After 20 hr, replace the medium with 8 ml complete DMEM.

   Gently add the medium to the side wall of the wells so as not to detach cells.

   Successfully transfected cells should show slight shrinkage and clustering, a good sign for effective lentivirus production (Fig. 1).

8. Incubate the plate again at 37°C and 5% CO_2 for an additional 48 hr.

9. To harvest viral particles, proceed by gently pipetting virus-containing medium into a 15- or 50-ml Falcon tube.

The volume of medium will change depending on how many plates the user is harvesting; the harvest can be scaled by multiplying the transfection mix volume by the number of plates used.

10. Centrifuge virus-containing medium for 7 min at 500 × *g* to acquire pellet.

    There should be a visible white pellet toward the bottom of the tube.

11. Gently draw the supernatant into a pipet without disturbing the cell pellet and filter through a 0.45-μm syringe filter using a 10-ml syringe into another 15- or 50-ml Falcon tube. Discard the pellet.

12. Measure virus concentration using Lenti-X GoStix Plus lentiviral titer kit (see Support Protocol).

    The next step is to concentrate and then store the virus at −80°C (see steps 13 to 20; recommended).

13. Mix the filtered supernatant with Lenti-X concentrator at a 3:1 ratio of filtered virus medium/concentrator.

14. Incubate the sample on ice for ≥1 hr.

15. Centrifuge concentrated medium for 45 min at 1500 × *g*, 4°C.

    A viral pellet should be visible after centrifugation.

16. Remove supernatant without disturbing the pellet.

17. Add complete medium of the user's choice or DPBS to the pellet at 1/10 or 1/100 of the original supernatant volume.

    The complete medium should be that of the target cells to be transduced later by the user.

    The dilution varies depending on the user's needs.

18. Gently resuspend the viral pellet by pipetting up and down 50 times without forming bubbles.

19. Once the viral pellet is resuspended, measure the concentration of the virus using the Lenti-X GoStix Plus lentiviral titer kit (see Support Protocol).

    The concentrated virus should be diluted 10- to 100-fold before loading into the titer kit to avoid saturation.

20. Store product for 2 to 4 months at −80°C. Thaw when needed.

    Please see information about sample data in the Understanding Results section.

QUANTIFICATION OF THE TITER OF LENTIVIRUS

SUPPORT PROTOCOL

This support protocol lists steps to quantify the titer of lentivirus from Basic Protocol 1. Follow the guide on Takara's website for the Lenti-X GoStix Plus lentiviral titer kit.

Materials

Lenti-X GoStix Plus lentiviral titer kit (Takara, cat. no. 631280), containing cassette and chase buffer
Diluted virus sample (from concentrated virus from Basic Protocol 1)

Smartphone

1. Download the GoStix Plus app (available on Google Play or in the Apple App Store by searching "GoStix Plus" in the search bar) onto a smartphone.

2. Optional: Add an email address to have the result sent to that address.
3. Enter the lot number by scanning the QR code on the Lenti-X GoStix Plus lentiviral titer kit foil pouch. Press the QR code icon to activate the scanner.

   The user can also enter the lot number manually.
4. Enter the number of tests to be scanned (1 to 8 tests).
5. Enter the sample names and the appropriate dilutions and then click "start."
6. Following the prompts within the app, add 20 µl diluted virus sample to the cassette from the kit.

   Dilution may be needed. See the Troubleshooting section for more information.
7. Add 80 µl chase buffer to the sample well and wait for 10 min using the timer in the app.
8. Ensure that the control band appears on the left and the test band appears on the right.
9. Using the app, align the cassette using the alignment tool.

   Proper alignment and focal length for imaging are achieved by using the outline of the cassette in the scanning window.

   Once proper alignment is achieved, the outline will turn green, and the cassette will automatically be scanned to measure the intensity of each band.
10. Once all samples are scanned, view the results in the "Result detail" window. Press "done" if all samples have been scanned.
11. Interpret the results.

    A discussion of the interpretation of results can be found on Takara's website (https://www.takarabio.com/learning-centers/gene-function/viral-transduction/lentivirus/lenti-x-gostix-plus-faqs). The GoStix Value (GV) units given to the user quantify the concentration of p24 protein in nanograms. Please visit takarabio.com/gostixhelp for additional guidance on retrieving downloaded results.

    Please see information about sample data in the Understanding Results section.

BASIC PROTOCOL 2

CULTURING, CHEMICAL TRANSFECTION, AND LENTIVIRUS TRANSDUCTION OF HT-29 CELLS

This protocol outlines the steps to culture the colorectal HT-29 cell line and perform chemical transfection or lentivirus transduction. A sufficient gene delivery rate can be achieved in HEK293T cells by either chemical transfection or lentiviral transduction. However, HT-29 cells require lentiviral transduction for effective gene delivery (Fig. 2).

Materials

HT-29 cells (ATCC, cat. no. HTB-38)
Complete HT-29 cell culture medium (see recipe), 37°C
Optogenetic RIPK3 (fused with mCherry) plasmid
Opti-MEM medium (Thermo Fisher, cat. no. 31985062)
PEI MAX (Polysciences, cat. no. 24765-1)
Concentrated and quantified La-RIPK3 virus (see Basic Protocol 1 and Support Protocol)
Polybrene transfection reagent
70% (v/v) isopropyl alcohol

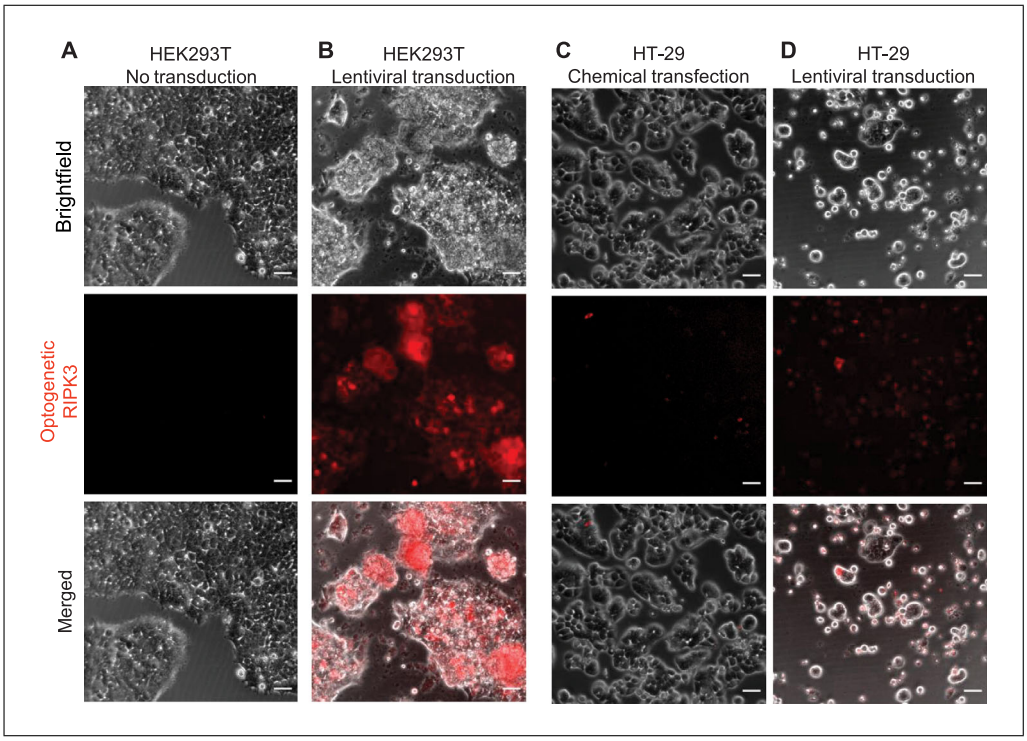

Figure 2 Lentiviral transduction provides a more effective gene delivery rate than chemical transfection. (**A**) HEK293T cells were treated with complete medium supplemented with polybrene (8 μg/ml). (**B**) HEK293T cells were treated with complete medium supplemented with polybrene (8 μg/ml) and the optogenetic RIPK3 lentivirus after the original virus-containing medium was concentrated 10 times. (**C**) HT-29 cells were transiently transfected with non-viral optogenetic RIPK3 plasmid, and images were taken 24 hr post-transfection. (**D**) HT-29 cells were transduced with lentivirus supplemented with polybrene (8 μg/ml), and images were taken 96 hr post-treatment. Scale bars, 50 μm.

60-mm culture plates (Fisher Scientific, cat. no. FB012921)
24-well culture plates (Fisherbrand, cat. no. FB012929)
Standard tabletop centrifuge (Sorvall Legend XTR) with swinging-bucket rotor (Thermo Scientific, TX-1000) and plate adapter
Parafilm
Large Kimwipes

Cell culture

1. Culture HT-29 cells in 4 ml complete HT-29 cell culture medium in a 60-mm culture plate at an initial seeding density of 2×10^6 cells, changing the medium every 3 days and splitting the cells when they are 80% confluent.

Chemical transfection

2a. Plate 1.6×10^5 cells/ml in a 24-well culture plate for chemical transfection.

Use a 300-μl seeding volume per well to have a more even distribution of cells within each well of the 24-well plate.

3a. Incubate the cells for 2 days. Supplement them with 500 μl fresh complete HT-29 cell culture medium 24 hr after cell plating.

4a. Gently remove the medium and add 450 μl fresh complete HT-29 cell culture medium before transfection.

5a. Prepare transfection mix using 500 ng of the optogenetic RIPK3 (fused with mCherry) plasmid 1.5 μl PEI MAX, and Opti-MEM medium to 50 μl.

6a. Incubate the transfection mix for 15 min at room temperature.

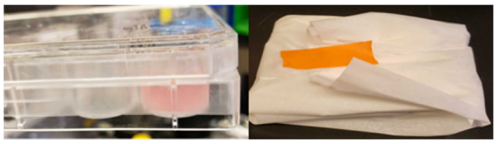

Figure 3 Setup for spinning down tissue culture plates. The tissue culture plate is wrapped before spinning to keep humidity and CO_2 in the wells.

7a. Add 50 µl transfection mix to each well dropwise and incubate the cells for 4 hr.

Gently rock the plate to facilitate the diffusion of the mix to the cells.

8a. After 4 hr, remove the transfection medium and add 500 µl fresh complete HT-29 cell culture medium to the cells.

Chemically transfected cells should show fluorescence from mCherry (Fig. 2C).

Lentivirus transduction

2b. Plate 1.6×10^5 cells/ml in a 24-well culture plate for lentivirus transduction.

3b. Pre-warm the centrifuge with a swinging-bucket rotor to 30°C.

To preset the pre-warming temperature, hold the "temp" button on the Sorvall Legend XTR for a few seconds and then set the temperature to 30°C. Click "start," and the centrifuge will start to rotate to warm it up. It will take about 30 to 60 min to pre-warm the centrifuge to 30°C. Once the warming is done, the instrument makes a beeping sound.

4b. Make a virus cocktail to be added to the target cells.

i. Concentrated and quantified La-RIPK3 virus amount: 1501 GV.

GV units are custom units from Takara and are ng/ml of the p24 protein of the lentivirus capsid.

This procedure is for 24-well plates. Ideally, the amount of virus should be increased or decreased based on the area of the plate.

ii. Polybrene transfection reagent: final concentration 8 µg/ml.

iii. Make up the rest of the volume with the complete medium of the target cells.

5b. Add the virus cocktail to the HT-29 cells in the 24-well plate (see step 2b).

6b. Incubate the cells in the 37°C incubator for 10 min.

7b. Proceed to spin infection:

i. Sterilize the centrifuge plate adapter with 70% isopropyl alcohol.

ii. Parafilm the entire junction of the cell plate and its lid and then wrap it with a large Kimwipe (Fig. 3).

This is done so the CO_2 and humidity can be maintained better during the 1-hr centrifugation (see step 7b, iii). Wrapping the plate using a Kimwipe minimizes the chance of contamination by bacteria likely to be present in the centrifuge.

iii. Place the Kimwipe-wrapped plate into the centrifuge adapter and balance the other side of the rotor with a plate of similar weight (within 1 g of difference).

iv. Proceed to centrifugation for 1 hr at 3000 rpm in a TX-1000 swinging-bucket rotor, 30°C.

Spin infection can improve the viral transduction of certain cells (this is mandatory for HT-29 cells).

8b. Once centrifugation is done, remove the Parafilm and return the plate to the incubator.

9b. Replace medium with 500 µl fresh complete medium 24 hr post-transduction.

Expression of optogenetic RIPK3 can be examined by measuring the fluorescence of mCherry.

Note that different cell lines show significant variation in the transduction rate. For example, HEK293T cells (Fig. 2D) show a higher transduction rate compared with HT-29 cells (Fig. 2B).

Please see information about sample data in the Understanding Results section.

OPTIMIZATION OF OPTOGENETIC STIMULATION CONDITIONS

BASIC PROTOCOL 3

This protocol lists steps to optimize the optogenetic stimulation conditions for minimal phototoxicity.

Materials

Stable HT-29 cells expressing the optogenetic RIPK3 system (see Basic Protocol 2)
SYTOX Green (Thermo Fisher, cat. no. S7020; see Basic Protocol 4, step 3)

Environmental control enclosure system (In Vivo Scientific)
Inverted microscope (Leica DMI8)
Imaging software (Leica LAS-X, Micromanager 1.4)

1. Treat the stable HT-29 cells expressing the optogenetic RIPK3 system with 100 µl SYTOX Green per well for 10 min in a sterile biosafety cabinet.

   The final concentration of SYTOX Green (which stains lytic cells) should be 0.5 µM in each well.

2. Turn on the environmental control enclosure system (5% CO_2 and 37°C; Fig. 4A) 1 hr before imaging. Adjust the flow rate to 300 cm^3/min (ccm).

   This helps to avoid water condensation during imaging.

3. Turn on the inverted microscope shortly before imaging (see Fig. 4 for setup of hardware and software).

4. Set the fluorescence intensity manager (Fig. 4B) at the second position.

5. Use a 10× objective and set the plate of stable HT-29 cells expressing the optogenetic RIPK3 system under the microscope.

6. Set up imaging parameters in imaging software as follows:

   a. Texas red (TXR): 100 ms.

      Purpose: To identify cells expressing the optogenetic RIPK3 system with mCherry.

   b. GFP: 15 ms.

      Purpose: To capture SytoxGreen-stained lytic cells and provide optogenetic stimulation.

   c. GFP: 80 ms.

      Purpose: To provide an extra dose of light for optogenetic stimulation. Two-step GFP stimulation is used so that signals for SytoxGreen-stained cells will be saturated and sufficient blue light is supplied for optogenetic stimulation.

   d. Brightfield: 100 ms.

      Purpose: To capture cell morphology.

   e. Dark interval: 5 min.

      Purpose: To minimize phototoxicity.

   f. Total imaging acquisition time: 20 hr.

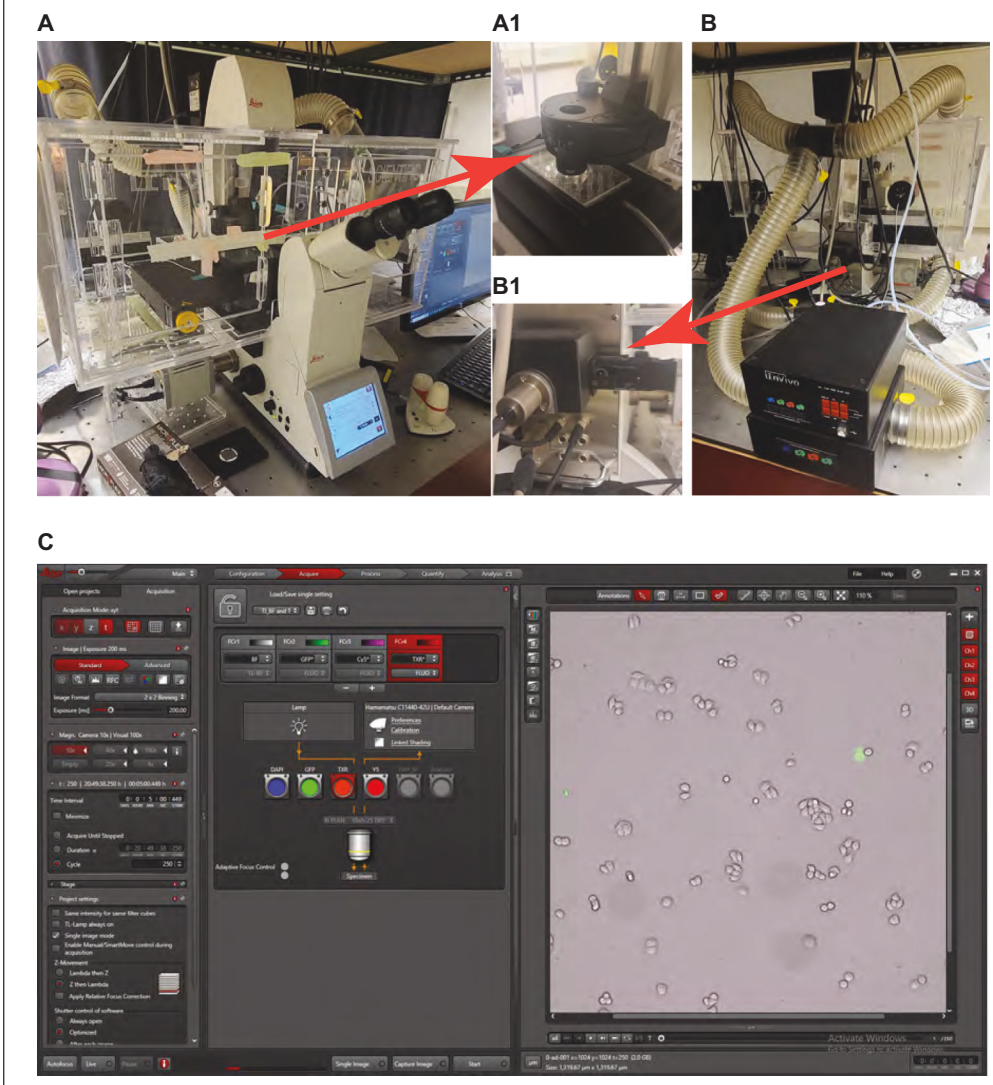

Figure 4 Hardware and software for acquiring images of optogenetic induction of RIPK3-mediated necroptosis. (**A**) Side view of the microscope equipped with an environmental control chamber that tunes the temperature and CO_2 concentration for long-term live-cell imaging. A1, Sample holder and a plate with cultured cells. (**B**) Rear view of the microscope, highlighting the tubes and CO_2 regulator. B1, Fluorescence intensity manager (FIM) and the liquid guide for light delivery. (**C**) Software interface for data acquisition.

7. Examine cell membrane rupture.

   Less than 5% cell membrane rupture should occur in wild-type cells. If a more vulnerable cell type is used, reduce the power and exposure time of the excitation light.

BASIC PROTOCOL 4

TIME-STAMPED LIVE-CELL IMAGING OF HT-29 LYTIC CELL DEATH

This protocol outlines the steps to achieve time-stamped live-cell imaging that captures HT-29 lytic cell death.

Materials

Stable HT-29 cells expressing the optogenetic RIPK3 system (see Basic Protocol 2)
Wild-type HT-29 cells (ATCC, cat. no. HTB-38)
Complete HT-29 cell culture medium (see recipe), 37°C
SYTOX Green (Thermo Fisher, cat. no. S7020)

12-well culture plates (Fisherbrand)
Inverted microscope (Leica DMI8) with optimized optogenetic stimulation conditions (see Basic Protocol 3)
Imaging software (Leica LAS-X, Micromanager 1.4.)
ImageJ software

1. Plate two wells of stable HT-29 cells expressing the optogenetic RIPK3 system and two wells of wild-type HT-29 cells (Gene-/Dark, Gene-/Light, Gene+/Dark, and Gene+/Light) in complete HT-29 cell culture medium in a 12-well culture plate at a seeding density of 0.25×10^6 cells per well and incubate for 24 hr in a 37°C and 5% CO_2 incubator.
2. Remove old medium from each well and wash away dead cells three times using fresh medium. Then, add 900 µl fresh medium to the cells and incubate for 30 min at 37°C and 5% CO_2.

   Before aspirating old medium from each well, tilt the 12-well plate at a 45° angle and move it in a circular motion to bring down any dead cells. Draw out the medium while holding the plate tilted, using a vacuum aspirator to ensure the dead cells are removed. Repeat the wash three times using fresh medium to completely remove any dead cells.

3. Stain the cells with 100 µl SYTOX Green per well for 10 min at room temperature in a sterile biosafety cabinet.

   The final concentration of SYTOX Green (which stains lytic cells) should be 0.5 µM in each well.

4. Start the imaging software and create a new project to be saved as an LIF file.
5. Proceed with live-cell imaging under the inverted microscope with optimized optogenetic stimulation conditions (see Basic Protocol 3), including the following imaging parameters:

   a. TXR: 100 ms.
   b. GFP: 15 ms.
   c. GFP: 80 ms.
   d. Brightfield: 100 ms.
   e. Dark interval: 5 min.
   f. Fluorescence intensity attenuator: position 2.
   g. Total data acquisition time: 20 hr.

6. Remove the plate after 20 hr of imaging, save the project LIF file (or TIFF stack of images), and analyze data using ImageJ software.

QUANTIFICATION OF HT-29 LYTIC CELL DEATH

BASIC PROTOCOL 5

This protocol outlines the data analysis steps to quantify HT-29 lytic cell death.

Materials

FIJI (*https://imagej.net/software/fiji/*)
CellProfiler (*https://cellprofiler.org*)
Desktop computer (computer with ≥16 GB RAM and fast CPU; preferred, Intel i7 or AMD Ryzen 7 equivalent)
TIFF stack acquired from microscope software (see Basic Protocol 4)
Microsoft Excel or equivalent quantification software (Matlab, Origin)

1. Open up FIJI and CellProfiler on a desktop computer.
2. Using FIJI, open up the TIFF stack acquired from microscope software by selecting File > Open > Browse.

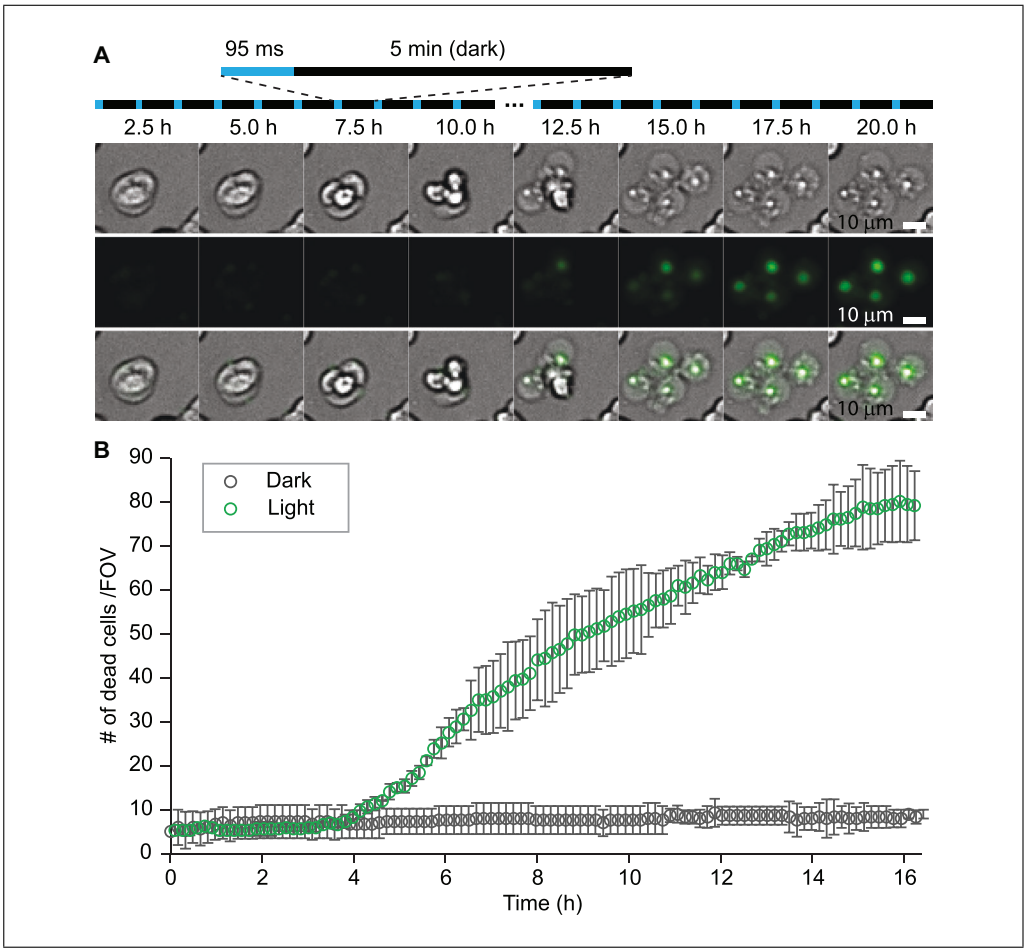

Figure 5 Time-stamped images of optogenetic induction of RIPK3-mediated lytic cell death. (**A**) HT-29 cells were transduced with lentivirus encoding the optogenetic RIPK3 system and cultured in an environmental control chamber. Blue light stimulation causes RIPK3-containing necrosome formation and membrane rupture. SYTOX Green stain was used to stain lytic cells. A pulse of 95-ms blue light stimulation was applied every 5 min, and cell morphology and staining were monitored. Representative images of a cluster of cells undergoing necroptosis are shown. Cell death proceeds through a burst and flattening of cell membranes concurrently stained by SYTOX Green. (**B**) Time-dependent cell death in dark and light conditions.

3. If the option is available, check the box "Open Virtual Stack" to reduce the amount of RAM used. Proceed by clicking "Okay".
4. Select Image > Color > Split Channels to split the individual color channels into Bright Field (Grey), SytoxGreen (Green), and mCherry-labeled Lentivirus (Texas Red).
5. Save each color channel separately in Image Sequence format by clicking File > Save as > Image Sequence (for easy documentation, these should be saved to a separate folder). Save representative cell death images by cropping the stack images (Fig. 5A).
6. For all constructs needing to be analyzed, open and organize all files in the same manner as in steps 2 to 5 for easy identification in CellProfiler.

Channels can be merged back by selecting Image > Color > Merge Channels and then clicking the corresponding color needed to merge for each color. For example, if the user wants to overlay a brightfield image with SytoxGreen only, they will select the brightfield TIFF stack as the "Grey" channel and the SytoxGreen TIFF stack as "Green". Then, the movie can be saved in the format the user desires through the same navigation as in step 5.

The GFP image with 15-ms exposure time is preferred for image analysis to avoid signal saturation. If the fluorescence signal is too weak, consider using the image with 80-ms exposure time.

7. Using CellProfiler, open an Image Sequence file previously created in step 5 by dragging the folder into CellProfiler.
8. In the Adjust Modules panel, click the "+" icon and search for IdentifyPrimaryObjects. Double-click to add a module.
9. In the same panel, search for ExportToSpreadsheet. Double-click to add a module.
10. In IdentifyPrimaryObjects, adjust the following parameters:
    a. Use Advanced Settings by clicking "Yes".
    b. Select the input image stack previously created in step 7.
    c. Adjust the typical diameter size of the cells to be analyzed.

    For HT29 cells, this value could range between 10 (min) to 20 (max) μm.

    d. Use the "Otsu" thresholding method.
    e. Adjust thresholding correction value to between 0.5 and 1.

    See Troubleshooting for why this is an important value.

    f. Allow all other settings to be the default.
11. In ExportToSpreadsheet, use the following settings:
    a. Direct the output file to the desired folder.
    b. Double-check that "Comma" is selected for column delimiter.
    c. Allow all other settings to be the default unless changes are desired.
12. Once all settings are completed, click "Analyze Images" to start the analysis.

    The progress bar on the bottom will show how many images CellProfiler has left to analyze before saving all data to the exported spreadsheet.
13. Repeat steps 7 to 12 for all other sets of images in three biological replicates.
14. Open the CSV file generated by CellProfiler into Microsoft Excel or equivalent quantification software to further analyze the data into a time series or to perform other quantification methods.

    For example, if the user wants to quantify the percentage of cell death, the following equation can be used in Excel:

$$= \frac{\#\ of\ SytoxGreen\ cells}{\#\ of\ mCherry\ Cells} \times 100\%$$

15. Plot the time-dependent cell death from the compiled results (Fig. 5B).

    Please see information about sample data in the Understanding Results section.

REAGENTS AND SOLUTIONS

Complete DMEM

DMEM (Corning, cat. no. 10-013-CV)
10% (v/v) FBS (Sigma-Aldrich)
1× penicillin/streptomycin (10,000 U/ml stock; Corning, cat. no. 30-002-CI)
Store ≤1 week at 4°C

Complete HT-29 cell culture medium

McCoy's 5A medium (ATCC, cat. no. 30-2007)
10% (v/v) FBS (Sigma-Aldrich)

1× penicillin/streptomycin (10,000 U/ml stock; Corning, cat. no. 30-002-CI)
Store ≤1 week at 4°C

COMMENTARY

Background Information

Well-established genetic and cell biological strategies have played a pivotal role in the study of cell death pathways. These previous studies provide the foundation for the design of optogenetic systems. Optogenetic stimulation provides high resolution in spatial and temporal control of cell signaling and therefore could provide new insights into the dynamic nature of cell death pathways. However, careful design of optogenetic experiments is warranted to verify the effect of the optogenetic response.

Critical Parameters

Gene delivery methods: Chemical versus lentivirus

Two commonly used gene delivery methods are chemical transfection and lentiviral transduction (see Basic Protocol 2). Chemical transfection uses cationic molecules that can partially neutralize the negative charge of DNA plasmids, which facilitates transport through the plasma membrane. Lentivirus packages the encoding genes in the transfer plasmid and forms non-propagated viral particles that infect the target cells. Chemical transfection can be done within 2 to 4 hr. Gene expression starts at ∼6 hr post-transfection. Lentivirus requires initial transfection in host cells (typically HEK293T cells), collection of the conditioned medium in 48 to 72 hr, and purification and concentration if needed. Upon transduction, gene expression takes another 3 days to 1 week.

The choice of gene delivery method depends on the cell model and the purpose of the application. Chemical transfection, despite its convenience and fast timeline, cannot provide sufficient transfection efficiency for all cell lines. In extremely challenging cell lines like HT-29 cells or primary culture, the transfection efficiency could be as low as 1%. Additionally, the gene expression profile is extremely heterogeneous. Lentiviral transduction provides a titer-dependent transduction rate. In our experience, the HT-29 transduction rate can reach 40% to 50% with lentiviral transduction. The expression profile from lentiviral transduction is more homogeneous. Whereas chemical transfection might be sufficient if the purpose is to observe single-cell behavior (e.g., through imaging), biochemical and cell biological analysis of cells (e.g., phosphorylation of target proteins or metabolic responses) may benefit from transduction from lentivirus or other viral vectors.

Choice of optogenetic systems and optimization of stimulation conditions

When working with a new cell or tissue model, optogenetic stimulations should be optimized to minimize phototoxicity (Basic Protocol 3). Commonly used optogenetic systems use visible light as a stimulating source. Light with shorter wavelengths introduces greater phototoxicity than that with longer wavelengths. Additionally, longer-wavelength light has a slightly deeper penetration depth than shorter-wavelength light and therefore is preferred for *in vivo* studies. However, different optogenetic systems also show different light sensitivity, e.g., different light-to-dark dynamic ranges or Kd ranges. Thus, the efficacy needs to be individually tested. Once a working system is confirmed, optical stimulation conditions should be optimized. Continuous exposure will introduce greater phototoxicity. Thus, an intermittent stimulation pattern could be used. The bottom line is to identify a safe range of experimental parameters, including wavelength, instantaneous light power, and total dosage, that do not introduce phototoxicity to wild-type cells.

Necessary controls

Whenever possible, optogenetic experiments (Basic Protocol 4) should be carried out with quadrant conditions in mind. Three controls are needed to delineate the optogenetic response, including Gene-/Dark, Gene-/Light, and Gene+/Dark. Only when all three controls show distinct cell responses from Gene+/Light could one safely interpret the observed phenotype as an optogenetic response. Figure 6 shows the distinct plasma membrane rupture phenotype in wild-type (non-transfection), mCherry-CRY2clust-transfected (without RIPK3), and optogenetic RIPK3–transfected cells after blue light stimulation. As expected, wild-type and mCherry-CRY2clust-transfected HT29 cells were not stained by SYTOX Green, indicating intact membranes, whereas optogenetic

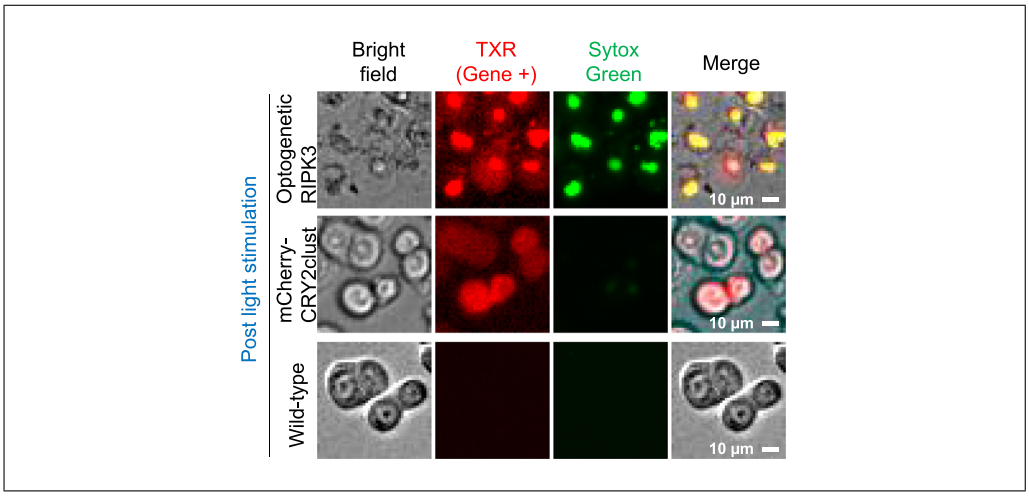

Figure 6 Representative images of HT-29 cells 20 hr after blue light stimulation under each condition. Optogenetic RIPK3–transfected cells (top) undergo blue light–inducible lytic cell death, which is confirmed by SYTOX Green staining. mCherry-CRY2clust-transfected (no RIPK3, middle) and wild-type (no transfection, bottom) cells are not stained, indicating intact membranes.

Table 1 Troubleshooting Guide for All Basic Protocols

Problem	Possible cause	Solution
Cells are over-confluent in the culture plate after adding the transfection mix	DMEM with FBS was used	Remember to use DMEM without FBS for a reduction in cell growth during the transfection period.
Poor transfection efficiency	There are many possible reasons for this: low-confluent cells; unhealthy cells; wrong amounts of transfer, packaging, and envelope plasmids; poor centrifugation; and/or inadequate cell conditions	Follow the proper molar ratio of plasmids and adhere to incubation times. Take pictures of any viral pellet found after concentrating for further verification that the transfection was successful.
Loss of viral product after concentrating	Incubation without ice or not incubated long enough	Incubate for >1 hr. Use ice during incubation.
No pellet seen after concentrating	Inadequate incubation time after mixing the virus with concentrator	Incubate the virus and concentrator for a longer time.
No cells were identified in CellProfiler; fewer green objects were identified, and more yellow/magenta objects were identified	The thresholding correction factor will help CellProfiler differentiate a cell from the background. If the thresholding factor is too low, CellProfiler cannot differentiate. However, the thresholding factor cannot be too high because CellProfiler will misidentify objects in the frame that are not cells.	Adjust the thresholding correction factor according to each image stack. This will require some trial and error.

RIPK3–transfected cells were clearly stained by SYTOX Green.

Real-time monitoring of cell response and healthiness

For long-term illumination, one needs an environmental chamber (Basic Protocols 3 and 4) that provides a sustained temperature (37°C) and acidity (5% CO_2) to ensure the healthiness of cells. Light sources from the optical microscope can be directly used to stimulate the optogenetic systems. Different channels of emission can be programmed together to monitor the cellular response to optogenetic stimulation in real time.

Troubleshooting

Common problems with the protocols, their causes, and potential solutions are shown in Table 1.

Understanding Results

Interpretation of optogenetic induction of lytic cell death requires data analysis of multicolor fluorescence images (Basic Protocol 5). Using CellProfiler, the number of dead cells versus time is shown (Fig. 5B). With optogenetic blue light simulation, the amount of cell deaths increases over time. When compared to control experiments undergoing no stimulation, the number of dead cells remains constant. One can compare the amount of SytoxGreen versus mCherry signal to account for the percentage of cell death by necroptosis.

Lentiviral transduction (Basic Protocol 2) is crucial for effective gene delivery. When producing lentivirus (Basic Protocol 1), a good benchmark for sufficient yield is the morphology of host cells (HEK293T) after transfection with the mix of transfer, packaging, and envelope plasmids. Clustering and shrinkage of cells are signs of effective transfection and production of lentivirus particles (Fig. 1). Because different cell lines show significant variation in the transduction rate, lentivirus transduction becomes mandatory for cell lines like HT-29 cells (Fig. 2). Fluorescence imaging provides a quick benchmark for transgene expression. Biochemical analysis, such as western blot or immunofluorescence staining, can be used to validate the expression level. Practically, we found that spinning down the cells together with lentivirus increased the transduction rate, but this procedure needs to be optimized for different cell lines.

Long-term imaging (Basic Protocol 4) tracks the cell response in real time and is selected as the primary way to quantify cell death in this article. A full environmental control enclosure system (Basic Protocols 3 and 4) is used to maintain the temperature, humidity, and CO_2 levels during data acquisition (Fig. 4A). Regular maintenance and checkups are warranted to ensure the appropriate functioning of the unit (e.g., the gas tank, CO_2 regulator, water batch, and temperature measurement). For data analysis (Basic Protocol 5), automated algorithms minimize human bias and are used in practice. Cells from different fluorescence channels are selected, and their intensity changes are recorded to calibrate cell death (Fig. 5). Cells under control conditions (e.g., wild-type, mCherry-CRY2clust) show a distinct cell death phenotype compared to optogenetic RIPK3–transfected cells (Fig. 6).

Time Considerations

Basic Protocol 1: Production of lentivirus encoding the optogenetic RIPK3 system: 3 days.

Basic Protocol 2: Culturing, chemical transfection, and lentivirus transduction of HT-29 cells: 3 days.

Basic Protocol 3: Optimization of optogenetic stimulation conditions: 24 hr.

Basic Protocol 4: Time-stamped live-cell imaging of HT-29 lytic cell death: 24 hr.

Basic Protocol 5: Quantification of HT-29 lytic cell death: 2 hr.

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Author Contributions

Teak-Jung Oh: Data curation; formal analysis; methodology; validation; writing—original draft. **Bryan Gworek:** Data curation; formal analysis; software; writing—original draft. **Amna Mehfooz:** Data curation; formal analysis; writing—original draft. **Kai Zhang:** Conceptualization; funding acquisition; project administration; resources; software; supervision; writing—original draft.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The complete raw data of time-stamped images of RIPK3-mediated lytic cell death are available from the corresponding author upon request.

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