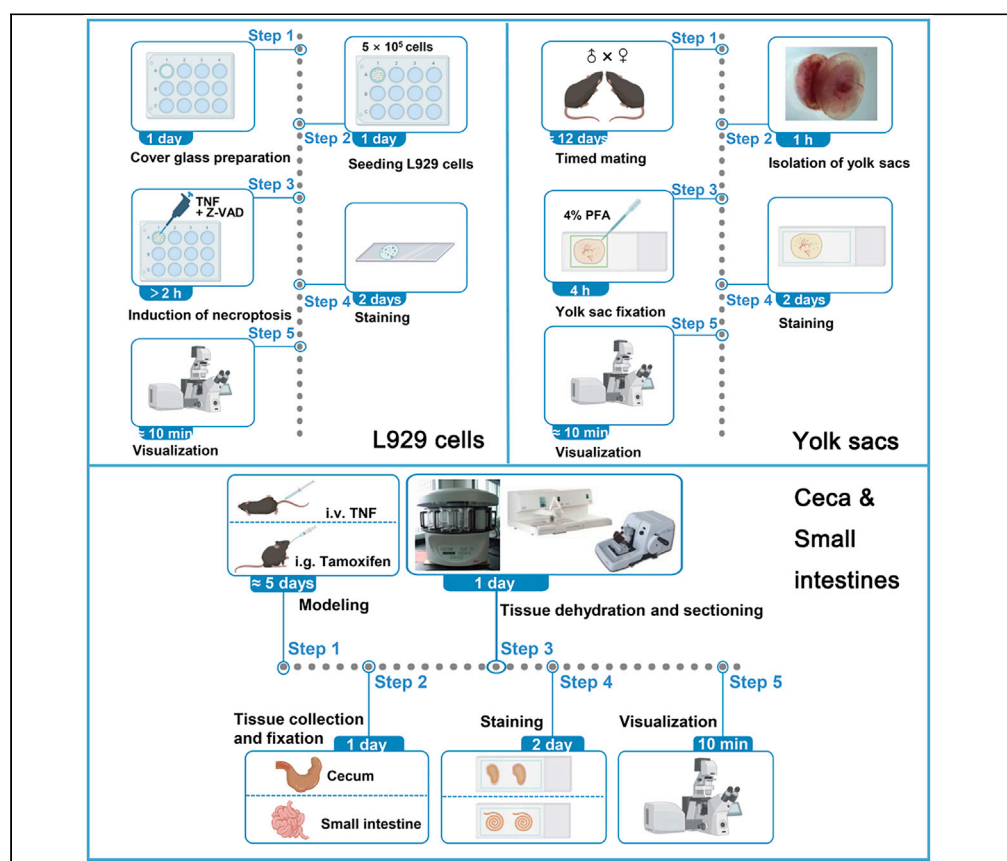


Protocol

Immunostaining of phospho-RIPK3 in L929 cells, murine yolk sacs, ceca, and small intestines



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Highlights

A detailed protocol for successful immunostaining of phosphorylated murine RIPK3

Specific conditions are provided for staining phospho-mRIPK3 in cells and tissues

This protocol is applicable to other murine cell lines and tissues such as livers

Though phospho-receptor-interacting protein 3 (RIP3 or RIPK3) antibodies are used in western blot, immunostaining of murine phospho-RIPK3 is challenging. Here, we verify and describe a detailed protocol for immunofluorescent detection of phospho-RIPK3 in L929 cells and mouse yolk sacs. We also describe in detail the model construction methods, sample preparation steps, and staining procedures for immunohistochemical labeling of RIPK3 activation in mouse ceca and small intestines by utilizing a specific commercially available antibody.

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

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Protocol

Immunostaining of phospho-RIPK3 in L929 cells, murine yolk sacs, ceca, and small intestines

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SUMMARY

Though phospho-receptor-interacting protein 3 (RIP3 or RIPK3) antibodies are used in western blot, immunostaining of murine phospho-RIPK3 is challenging. Here, we verify and describe a detailed protocol for immunofluorescent detection of phospho-RIPK3 in L929 cells and mouse yolk sacs. We also describe in detail the model construction methods, sample preparation steps, and staining procedures for immunohistochemical labeling of RIPK3 activation in mouse ceca and small intestines by utilizing a specific commercially available antibody. For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021) and Wang et al. (2020).

BEFORE YOU BEGIN

A complete list of required materials and equipment is provided in the [key resources table](#). Solutions should be prepared in advance according to the recipes and can be stored as indicated.

Institutional permissions

All mice used in this experiment were housed in specific pathogen-free condition with 12-h light/dark cycle and access to food and water ad libitum at Xiamen University Laboratory Animal Center. Animal husbandry and all mouse experiments were reviewed and approved by Laboratory Animal Management and Ethics Committee of Xiamen University (approval number XMULAC20180126) and were in strict accordance with good animal practice as defined by Xiamen University Laboratory Animal Center. For researchers who are about to use this protocol in mice, please acquire permissions from your institutions. All mouse husbandry and experiments should be reviewed and approved by the Laboratory Animal Management and Ethics Committee of your institute and should be in strict accordance with good animal practice as defined by the Laboratory Animal Center of your institute.

Cell lines

⌚ Timing: 1 day

Prepare wild-type L929 cells or counterparts for immunofluorescence staining in cells.



Mice

⌚ Timing: 8–12 weeks

For the yolk sac immunofluorescence staining, prepare *Casp8*^{+/-} male and female mice or similar mouse models that show defects caused by RIPK3 kinase-dependent necroptosis in yolk sacs (Zhang et al., 2021).

Prepare 8- to 12-week-old wild-type male or female mice on a C57BL/6 background for the induction of TNF-induced systemic inflammatory response syndrome (SIRS) and cecum staining.

For the small intestine staining, *Setdb1* conditional knockout mice kindly provided by Prof. Wei Mo (*Setdb1*^{fl/fl} Vil-CreERT2 (Wang et al., 2020)) were used as an example in this protocol but they can be replaced by any other RIPK3 kinase-involved inflammatory bowel disease (IBD) mouse models.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-RIPK3 (T231 + S232)	Clone 2D7, produced in-house and licensed to and now available from Abcam	Cat# ab205421
PECAM (CD31)	BD Biosciences	Cat# 550274
Chemicals, peptides, and recombinant proteins		
Mouse TNF α	Novoprotein (optional)	Cat# CF09
Pan Caspase Inhibitor Z-VAD-FMK	R&D	Cat# FMK001
Neutral balsam	Sinopharm Chemical Reagent Company (optional)	Cat# 10004160
Paraformaldehyde	Merck Millipore (optional)	Cat# 104005
Ethanol	Sinopharm Chemical Reagent Company (optional)	Cat# 10009218
Hematoxylin	Fuzhou Maixin Biotech Company (optional)	Cat# CTS-1096
Paraplast	Leica (optional)	Cat# 39601095
KH ₂ PO ₄	Sigma (optional)	Sigma (optional)
KCl	Sigma (optional)	Sigma (optional)
NaCl	Sigma (optional)	Cat# S5886
Sodium citrate (critical reagent)	Sigma	Cat# 71402
Tween-20	Sangon Biotech Company (optional)	Cat# TB0560
28% ammonia	Sinopharm Chemical Reagent Company (optional)	Cat# 10002118
Potassium dichromate	Sinopharm Chemical Reagent Company (optional)	Cat# 7778-50-9
Concentrated sulfuric acid	Sinopharm Chemical Reagent Company (optional)	Cat# 7664-93-9
Triton X-100	Sangon Biotech Company (optional)	Cat# A110694-0500
0.1% Gelatin Solution	Millipore	Cat# ES-006-B
Critical commercial assays		
BLOXALL® Blocking Solution	Vector Laboratories	Cat# PK-8200
Normal horse serum blocking solution (critical reagent)	Vector Laboratories	Cat# PK-8200
Goat serum	Jackson ImmunoResearch	Cat# 005-000-121
Bovine serum albumin (BSA), for biochemistry	VWR Chemicals	VWRC0332-100G
Horse Anti-Mouse/Rabbit IgG, Biotinylated	Vector Laboratories	Cat# PK-8200

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
VECTASTAIN Elite ABC Reagent	Vector Laboratories	Cat# PK-8200
ImmPACT DAB Peroxidase (HRP) Substrate (critical reagent)	Vector Laboratories	Cat# PK-8200
ProLong® Gold Antifade Reagent	Invitrogen	Cat# P36934
Hoechst 33342	Beyotime	Cat# C1022
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Invitrogen	Cat# A-11004
Alexa Fluor 488 goat anti-mouse antibody	Invitrogen	Cat# A11029
Alexa Fluor 594 goat anti-rat antibody	Invitrogen	Cat# A11007
Nail polish	Dreambooks	Dreambooks
Experimental models: Cell lines		
Mouse: L929 cells	ATCC	CCL-1
Experimental models: Organisms/strains		
Mouse: Casp8 ^{+/-} , 8–12 weeks, males and females	Zhang et al. (2021)	N/A
Mouse: C57BL/6J, 8–12 weeks, males and females	The Jackson Laboratory	JAX: 000664
Mouse: Setdb1 ^{fl/fl} Vil-CreERT2, 8–12 weeks, males and females	Wang et al. (2020)	N/A
Other		
Black western blot incubation box	LI-COR Biosciences (optional)	Cat# 929-97301
Scientific Cover glasses, F 20 mm	NEST (optional)	Cat# 801008
Parafilm	Bemis Company, Inc (optional)	Cat# PM996
Tissue cassettes	CITOGLAS (optional)	Cat# 80312-3161
Microtome	Leica (optional)	Cat# RM2016
Disposable blade	Leica (optional)	Cat# 819
Tissue section flotation bath	Changzhou Haosilin Medical Instrument Company (optional)	Cat# TEC2601
Slides	CITOGLAS (optional)	Cat# 188105W
Adhesion microscope slides	VWR	Cat# TOM-11/90
Light-protective slide holder	Haotian	Haotian
Slide drier	Changzhou Haosilin Medical Instrument Company (optional)	Cat# TEC2602
Microscope	Olympus (optional)	Cat# CX21
Leica DM4B	Leica (optional)	Cat# Leica DM4B
Embedding cassette	Beyotime (optional)	Cat# FSR902
Semi-enclosed benchtop tissue processor	Leica (optional)	Cat# TP1020
Super PAP pen	Fuzhou Maixin Biotech (optional)	Cat# PEN-0002
Cover glasses for tissue staining	Sail Brand	Sail Brand
The embedding workstation	Leica (optional)	Cat# EG1160
Shaker	Haimen Kylin-Bell Lab Instruments (optional)	Cat# TS-2
Pressure cooker	Guangdong Shunfa Hardware Products (optional)	Cat# SFYLG20UX
Electromagnetic oven	Midea Group (optional)	Cat# SK2105
Mouse tail vein injection instrument	Nanjing Karwin Biotechnology (optional)	Nanjing Karwin Biotechnology (optional)
Zeiss LSM 880+Airyscan	ZEISS	Cat# Zeiss LSM 880+Airyscan

Note: All the labeled optional equipment, software, and suppliers can be replaced by similar equipment, software, and suppliers.

MATERIALS AND EQUIPMENT

PBS preparation

⌚ Timing: ≈ 20 min

PBS	Final concentration	Amount
KH ₂ PO ₄	2 mM	0.27218 g
Na ₂ HPO ₄	10 mM	1.4196 g
NaCl	137 mM	8.00628 g
KCl	2.7 mM	0.201285 g
H ₂ O	n/a	Add to 1 L
Total	n/a	1 L

Note: PBS must be sterilized by autoclaving and can be stored at room temperature (25°C) for 6 months.

Wash buffer preparation

⌚ Timing: ≈ 5 min

Wash buffer	Final concentration	Amount
Tween-20	0.1%	1 mL
PBS	n/a	1 L
Total	n/a	1 L

Note: Wash buffer should be freshly prepared on the day of use.

4% paraformaldehyde (PFA) buffer preparation

⌚ Timing: ≈ 16 h

4% PFA buffer	Final concentration	Amount
PFA	4%	40 g
PBS	n/a	Add to 1 L
Total	n/a	1 L

Note: (1) 4% PFA buffer must be stored at 4°C for up to 1 month. (2) PFA and PBS mixture can be put in a 56°C oven overnight (about 16 h), which will make PFA easier to dissolve in PBS. 5–10 drops of 10 N NaOH can be added to allow PFA to dissolve but the final pH of 4% PFA solution must be adjusted to 7.4 with 1 N HCl. (3) PFA should be used in a fume hood to avoid eye and skin exposure as instructed by the manufacturers.

Citrate antigen retrieval buffer (pH 6.0) preparation

⌚ Timing: ≈ 20 min

Citrate antigen retrieval buffer	Final concentration	Amount
Sodium Citrate	1.0 mM	1.4705 g
Adjust the pH to 6.0 with 1 N HCl		
Tween-20	0.05%	0.25 mL
H ₂ O	n/a	Add to 0.5 L
Total	n/a	0.5 L

Note: Citrate antigen retrieval buffer should be freshly prepared on the day of use.

0.08% ammonia

⌚ Timing: ≈ 5 min

0.08% ammonia	Final concentration	Amount
28% ammonia	0.08%	3 mL
H ₂ O	n/a	Add to 1 L
Total	n/a	1 L

Note: 0.08% ammonia can be stored at room temperature (25°C) for 6 months.

Chromic acid preparation

⌚ Timing: ≈ 30 min

Chromic acid	Final concentration	Amount
Potassium dichromate	425 mM	5 kg
H ₂ O	n/a	30 L
Sulfuric acid	24.5%	10 L
Total	n/a	40 L

Note: Chromic acid can be stored at room temperature (25°C) for one month. Chromic acid is corrosive, so protective measures should be taken during configuration. Five kilograms of potassium dichromate should be dissolved in 30 L of water first. Sulfuric acid (10 L) should be added slowly and carefully to potassium dichromate solution (do not add water to sulfuric acid) and mixed evenly. Pour while mixing with a glass rod and pay special attention to avoid any splash.

Permeabilization buffer

⌚ Timing: ≈ 5 min

Permeabilization buffer	Final concentration	Amount
Triton X-100	0.25%	2.5 mL
PBS	n/a	1 L
Total	n/a	1 L

Note: Permeabilization buffer should be freshly prepared on the day of use.

STEP-BY-STEP METHOD DETAILS

Immunofluorescence in L929 cells

⌚ Timing: 4 days

1. Soak NEST Scientific cover glasses in chromic acid overnight and rinse them with fresh water on the next day. Washed cover glasses can be kept in 75% ethanol for a long period of time.

2. Using forceps, dip a single cover glass into 95%–100% ethanol. Blot off excess ethanol and then wave the cover glass through a flame. When flame extinguishes and no more ethanol remains, drop the now-sterile cover glass into one well of a 12-well plate.
3. Add 500 μ L of PBS to each well of the plate (cover glass should be totally immersed in the solution), and incubate the plate in a 37°C incubator for a minimum of 30 min to degas.
4. Remove PBS and add 500 μ L of 0.1% Gelatin Solution to each well of the plate. Incubate the plate in a 37°C incubator for 10 min and then wash once with 500 μ L of PBS.
5. Seed 5×10^5 L929 cells in each well and leave the plate in a 37°C incubator overnight.
6. On the next day, replace the medium with fresh medium containing TNF (20 μ M) and Z-VAD (20 μ M).
7. At each indicated time point (0, 2, or 3-h post treatment), aspirate the medium and wash each well once with 500 μ L of PBS.
8. Add 500 μ L of 4% PFA to each well and incubate at room temperature (25°C) for 15 min to fix the cell. Wash twice with PBS.
9. Add 500 μ L of 0.25% TritonX-100 to each well and incubate at room temperature for 10 min. Wash twice with PBS.
10. Add 500 μ L of 5% BSA to each well and incubate at room temperature for 1 h.
11. On a Parafilm in a light-protective black western blot incubation box, for each sample, add 50 μ L of 2.7 μ g/mL phospho-RIPK3 antibody in 5% BSA.

Note: Please pay attention that we use final concentrations for antibodies instead of dilutions in this protocol.

12. Take the cover glasses out of the 12-well plate using a syringe needle bent at the tip and a tweezer and then place the cover glass face down on the antibody droplet. Incubate at 4°C overnight ([Methods video S1](#)).

△ CRITICAL: When lifting the cover glasses, be careful not to scratch the cells and not to reverse the sides of the cover glasses. Place a 35-mm dish of PBS in the incubation box to ensure a moist condition if needed.

13. Place the cover glasses back into the 12-well plate, face up, and wash with 500 μ L of PBS three times for 5 min each.
14. Prepare the staining solution: 5% BSA added with 1:200 Goat anti-mouse IgG (H+L) Alexa Fluor 568 Antibody and 1 μ g/mL Hoechst.
15. Change Parafilm in the black incubation box and add 50 μ L of the staining solution onto the new Parafilm for each sample, place the cover glasses face down onto the antibody droplet and incubate in the dark at room temperature for 30 min.
16. Place the cover glasses back into the 12-well plate, face up, and wash with 500 μ L of PBS three times for 5 min each.
17. Add 9 μ L of ProLong® Gold Antifade Reagent onto CITOGLAS slides and carefully place the cover glasses face down onto the droplets. Use nail polish to seal the rim of the cover glasses and place the slides in a light-protective slide holder to dry overnight at room temperature.

△ CRITICAL: Avoid bubbles formed during this process. Seal the rim of the cover glasses completely using nail polish to prevent any leak.

18. Use Zeiss LSM 880+Airyscan laser-scanning confocal microscope to acquire images. At the channel list, select Alexa Fluor 568 for phospho-RIPK3 and Hoechst 33342 for Hoechst, and also take a bright-field image to show the overall morphology of the cells.

△ CRITICAL: Signals of phospho-RIPK3 exist in necrotic cells, hence, find necrotic cells first by morphology and then you will find phospho-RIPK3 signals easily ([troubleshooting 1 and 2](#)).

Immunofluorescence in yolk sacs

⌚ Timing: 14 days

19. Prepare the mice for timed mating: house 3- to 4-month-old *Casp8*^{+/-} males individually for 1–2 weeks prior to mating. Group house 8- to 15-week-old *Casp8*^{+/-} females for 10–14 days prior to mating and expose them to soiled bedding from a male's cage 2 days before mating.
20. Timed-mating: add 1–2 female(s) in estrus into each stud male's cage at 9:00 PM. Examine females for vaginal plugs and separate them from male mice at 9:00 AM the next morning. Set the morning when a vaginal plug is found as embryonic day 0.5 (E0.5).
21. At 9:00 AM in the morning of E10.5, euthanize pregnant females via CO₂ exposure for at least 5 min until no breathing is observed.
22. Ensure death by performing a toe pinch. Perform cervical dislocation as a secondary method of euthanasia.

Note: Yolk sacs should be collected before the *Casp8*^{+/-} embryos are resorbed.

23. Isolate embryos and separate the yolk sac from the embryo proper.
24. Mount yolk sacs on adhesion microscope slides. Allow the slides to dry at room temperature for 20 min.
25. Use a Super PAP Pen to draw a circle around the yolk sac on the slides to confine the flow of reagents added later to the defined area.

Note: It is critical to use adhesion microscope slides.

26. Fix the yolk sacs by adding 50 µL of 4% PFA in PBS for 4 h at 4°C ([troubleshooting 3](#)).
27. Aspirate 4% PFA, permeabilize the cells by adding 50 µL of the permeabilization buffer and incubating at room temperature for 45 min.
28. Discard the permeabilization buffer, add 50 µL of the blocking solution containing 2% goat serum in PBS, and incubate at room temperature for 1 h.
29. Aspirate the blocking solution, add 50 µL of the primary antibody cocktail (blocking solution added with 0.78 µg/mL rat anti-PECAM antibody and 2.7 µg/mL mouse anti-phospho-RIPK3 antibody), and incubate at 4°C overnight.
30. Wash the slides with 50 mL of PBS three times for 5 min each and then incubate for 1 h in the dark at room temperature with 50 µL of the secondary antibody cocktail (blocking solution containing 1:200 diluted Alexa Fluor 594 goat anti-rat antibody and 1:200 diluted Alexa Fluor 488 goat anti-mouse antibody).
31. Aspirate the secondary antibody solution, add 50 µL of 1 µg/mL Hoechst in PBS, incubate for 1 min at room temperature in the dark, and then wash the slides with 50 mL of PBS three times for 5 min each.
32. Add 9 µL of ProLong® Gold Antifade Reagent onto the yolk sac on the slide and carefully place one cover glass onto the droplet. Use nail polish to seal the rim of the cover glass and place the slide in a light-protective slide holder to dry overnight at room temperature.
33. Use Zeiss LSM 880+Airyscan laser-scanning confocal microscope to acquire images. At the channel list, select Alexa Fluor 488 for phospho-RIPK3, Alexa Fluor 594 for PECAM, and Hoechst 33342 for Hoechst.

TNF injection, tamoxifen treatment, tissue collection, fixation, and sectioning

⌚ Timing: 5 or 7 days

34. Detailed protocol for TNF injection and cecum collection has been described elsewhere ([He et al., 2021](#)). Briefly,

- a. inject the mice intravenously with murine TNF α 300 μ g per kg body weight (μ g/kg) prepared in endotoxin-free saline.
 - b. harvest ceca at 8-h post-injection and fix them in 4% PFA overnight ([troubleshooting 4](#)).
 - c. clean feces after fixation.
- For the collection of small intestines,
- d. treat the *Setdb1^{fl/fl}* mice and the *Setdb1^{fl/fl}* Vil-CreERT2 mice with one dose of 400 mg/kg tamoxifen by gavage,
 - e. collect small intestines at day 4 post tamoxifen injection,
 - f. clean the feces, roll the small intestine up, and fix the tissues in 4% PFA (this can be any other mouse models with small intestine injury and inflammation).
35. Transfer the cecum and small intestine samples to the semi-enclosed benchtop tissue processor for dehydration: sequentially incubate them.
 - a. in 50%, 70%, and 80% ethanol in water (v/v) for 25 min,
 - b. in 95% ethanol in water (v/v) twice for 15 min each,
 - c. in 100% ethanol twice for 30 min each,
 - d. in ethanol and xylene mixture (1:1 v/v) for 30 min,
 - e. in xylene twice for 20 min each.
 - f. Finally, incubate them in paraplast tissue embedding medium twice at 60°C for 1.5 h each.
 36. Transfer the infiltrated tissues into the embedding workstation (working temperature: 60°C) and embed them with an appropriately sized embedding mold.
 37. Trim the tissue-embedding paraffin blocks and section the tissues at a thickness of 5 μ m on an RM2016 microtome.
 38. Float the tissue sections in a tissue section flotation bath and use CITOGlas slides to pick the tissue sections from the water bath.
 39. Dry the slides on a 42°C slide drier and then in a 56°C oven. For details of sectioning, please refer to ([He et al., 2021](#)).

Deparaffinization and immunohistochemistry

⌚ Timing: 2 days

40. Deparaffinization:
 - a. incubate the slides in xylene for 10 min,
 - b. repeat the xylene incubation twice,
 - c. move on to 100% ethanol for 10 min,
 - d. repeat the 100% ethanol incubation once,
 - e. and then proceed to 90% ethanol in water (v/v) for 5 min,
 - f. 80% ethanol in water (v/v) for 5 min,
 - g. 70% ethanol in water (v/v) for 5 min,
 - h. and wash the slides in ddH₂O for 5 min three times.

Note: Xylene and neutral balsam (which is dissolved in xylene and will be used later) should always be used with adequate ventilation, preferably in a fume hood. Eye and skin exposure should be avoided. Follow the chemical safety data sheet when handling these reagents.

41. Antigen retrieval: put slides in a pressure cooker containing antigen retrieval buffer and make sure the buffer covers the whole tissue. Place the pressure cooker on an induction stove and heat it at 1800 W. Let the cooker vent for 1.5 min and then turn the stove off. Allow the cooker to cool down to room temperature.
42. After antigen retrieval, use a Super PAP Pen to draw a circle around the tissue so that all reagents and antibodies except the washing solution will be added to area within the circle.

43. Wash the slides in wash buffer on a shaker at 55 rpm for 5 min, repeat the washing step twice, and incubate the slides in BLOXALL Blocking Solution at room temperature for 10 min.
44. Wash the slides using washing solution on a shaker for 5 min at 55 rpm three times, add 50 μ L of 2.5% horse serum to the tissue within the circle, and incubate at room temperature for 30 min.
45. Remove excessive serum from the slides and add 50 μ L of phospho-RIPK3 (T231 + S232) antibody in 2.5% horse serum (6.75 μ g/mL for ceca and 1.35 μ g/mL for small intestines). Put the slides into a slide rack and incubate at 4°C for 12 h.

Note: Put a small dish of water inside the slide rack so that the slides will not dry out.

46. After the phospho-RIPK3 antibody incubation, place the slide rack on the benchtop for about 30 min to warm to room temperature.
47. Wash the slides using wash buffer on a shaker at 55 rpm for 5 min. Repeat the wash step twice. Incubate the slides with 50 μ L of biotinylated horse anti-mouse/rabbit IgG secondary antibody at room temperature for 1 h.
48. Wash the slides using wash buffer on a shaker at 55 rpm for 5 min. Repeat the wash step twice. Incubate with VECTASTAIN Elite ABC Reagent at room temperature for 30 min.
49. After three washes using wash buffer on a shaker at 55 rpm for 5 min each, 1:1 mix the ImmPACT DAB EqV solutions as instructed by the manufacturer and incubate the slides until appropriate stain intensity develops.
50. Use ddH₂O to wash the slides 3 times, and wash for 5 min each time on a shaker at 55 rpm at room temperature.
51. Counterstain the slides with 50 μ L of Hematoxylin for 1.5 min at room temperature.
52. After 3 washes with ddH₂O for 10 s per wash, incubate the slides in 0.08% ammonia for 6 s and then wash 3 times with ddH₂O for 10 s per wash.
53. Dehydrate the slides with 80% ethanol in water (v/v) for 1 min, 90% ethanol for 1 min, 100% ethanol for 1 min twice, and 100% xylene for 1 min twice.
54. Add the permanent mounting medium neutral balsam to the sections, cover the sections with cover glasses, and press gently to get rid of any bubbles.

Note: Bubbles can easily form during this process. You can add neutral balsam onto the tissue, let one side of the cover glass touch the droplet first, and then slowly put the other side down to get rid of the bubble.

55. Let the slides dry in a fume hood at room temperature for 12 h.

▮▮ Pause point: These slides can be stored permanently at room temperature.

56. Use Leica DM4B to capture the images and analyze the data.

EXPECTED OUTCOMES

When necroptosis occurs, RIPK3 is phosphorylated, leading to MLKL phosphorylation, aggregation, and translocation to the plasma membrane to execute cell death.

As [Figure 1](#) showed, 2- or 3-h post-TNF α and Z-VAD-treatment, necroptotic wild-type L929 cells exhibited morphology changes and phospho-RIPK3 puncta in the cytosol. While in wild-type L929 cells without treatment or in *Ripk3*^{-/-} L929 cell treated with TNF α +Z-VAD for 3 h, there was no phospho-RIPK3 signal detected.

As shown in [Figure 2](#), RIPK3 phosphorylation was detected in *Casp8*^{-/-} yolk sac endothelial cells but not in wild-type yolk sac endothelial cells.

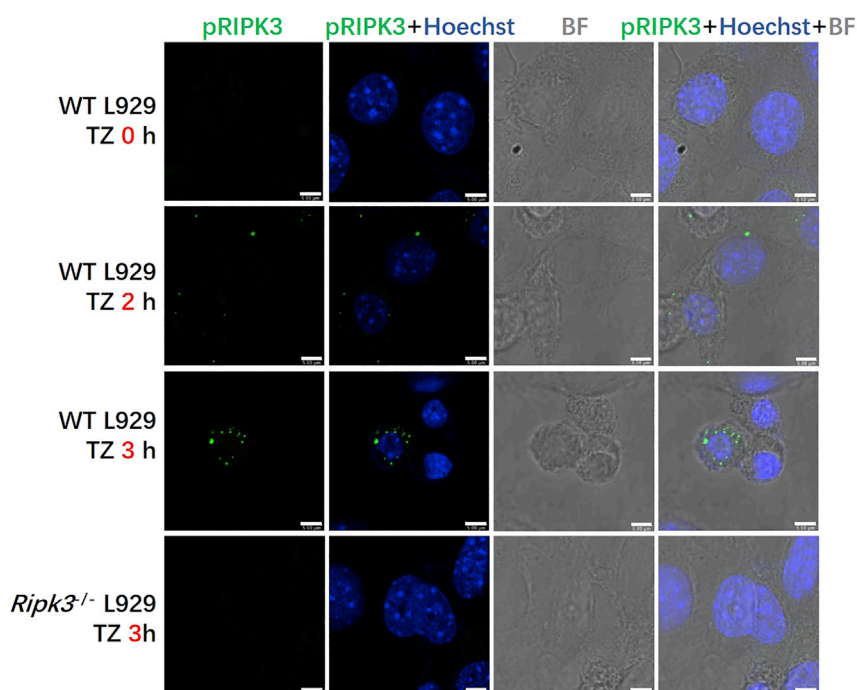


Figure 1. Immunofluorescence staining of phospho-RIPK3 in L929 cells

Wild-type or *Ripk3*^{-/-} L929 cells with or without TNF α (20 μ M) + Z-VAD (20 μ M) treatment were fixed and stained with anti-phospho-RIPK3 (2.7 μ g/mL) antibodies. Hoechst: 1 μ g/mL. Scale bar, 5 μ m.

Cecum is the most sensitive organ in TNF α -treated wild-type mice. Damage in cecum is blocked by the blockade of necroptosis (Yang et al., 2020; Chen et al., 2015; Deng et al., 2022; Cao et al., 2018). As Figure 3 showed, RIPK3 phosphorylation was remarkable in TNF α -treated cecum but not in the control cecum.

Setdb1 conditional knockout in intestinal epithelial cells leads to RIPK3-dependent necroptosis and inflammation in the gut (Wang et al., 2020). As shown in Figure 4, there were massive phospho-RIPK3 detected in the *Setdb1*-deficient ileum in sharp contrast to the control ileum.

LIMITATIONS

The only limitation of this protocol might be the availability of *Casp8*^{-/-} embryos and *Setdb1*^{fl/fl} Vil-CreERT2 mice but they can be replaced by any similar necroptosis-involved mouse models.

TROUBLESHOOTING

Described below are some potential problems and recommendations for troubleshooting.

Problem 1

Why can't I get any signals in the cells?

Potential solution

First, when you incubate the cells with primary antibody or secondary antibody, make sure not to reverse the sides of the cover glass. Second, the fluorescence of the secondary antibody is easy to quench so all steps following the addition of fluorescent secondary antibody should be done with care to avoid light.

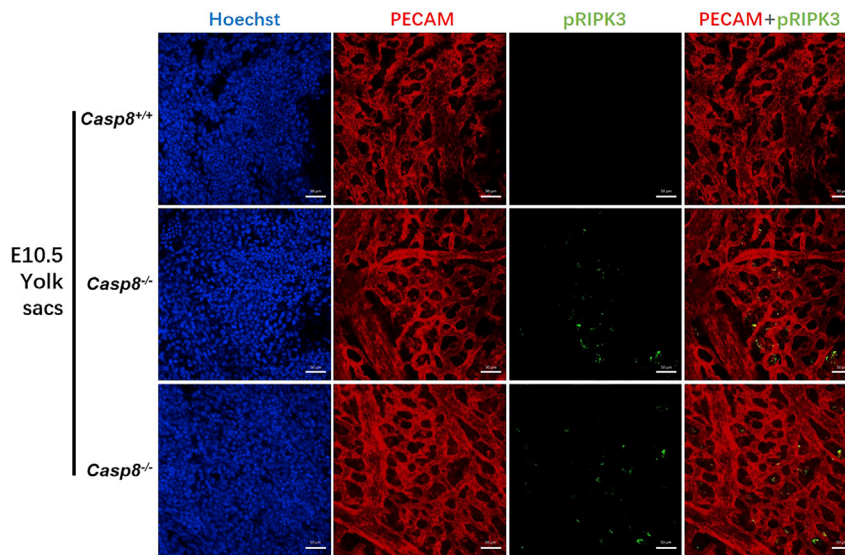


Figure 2. Immunofluorescence labeling of phospho-RIPK3 in yolk sacs

E10.5 yolk sacs of *Casp8*^{+/+} embryos and *Casp8*^{-/-} embryos were fixed and stained with anti-PECAM (0.78 µg/mL) and anti-phospho-RIPK3 (2.7 µg/mL) antibodies. Hoechst: 1 µg/mL. Scale bar, 50 µm.

Problem 2

Why do I have few cells on the cover glass?

Potential solution

First, incubate the cover glass with 0.1% Gelatin Solution for enough time at proper temperature (for example, for 1 h at 37°C). Second, when you take the cover glass out of the 12-well plate, clamp the edge of the cover glass. Third, wash steps must be done gently to avoid cells being washed away.

Problem 3

Why do the yolk sacs detach from the slides when I fix them?

Potential solution

Adhesion microscope slides must be used to mount the yolk sacs. Before you add 4% PFA onto the tissues, you must confirm that your tissues are dried and attached to the slides. Putting the slides in a fume hood can make the tissues dry faster.

Problem 4

Why can't I get damaged ceca in my SIRS model?

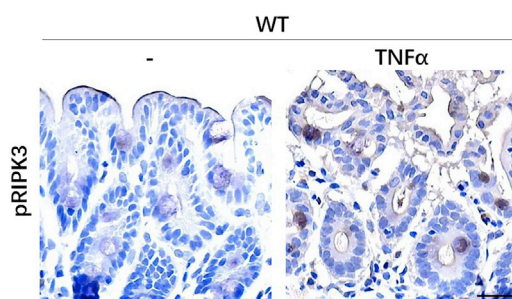


Figure 3. Immunohistochemical labeling of phospho-RIPK3 in ceca after TNF α injection

Ceca of WT mice with or without i.v. injection with 300 µg/kg TNF for 8 h were sectioned and stained with anti-phospho-RIPK3 antibody (6.75 µg/mL). Scale bar, 50 µm.

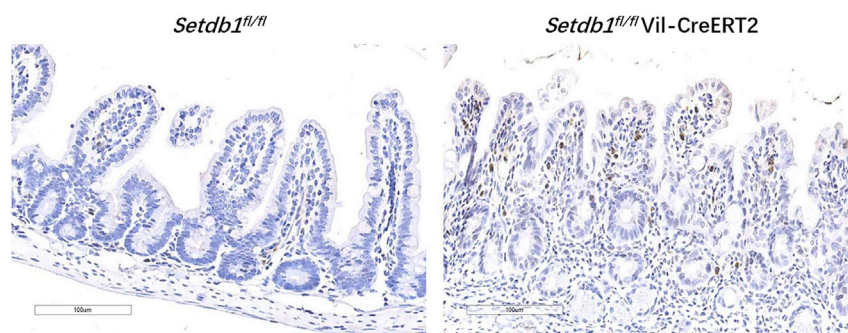


Figure 4. Immunohistochemical staining of phospho-RIPK3 in ilea

Ilea of *Setdb1^{fl/fl}* or *Setdb1^{fl/fl} Vil-CreERT2* mice treated with one dose of 400 mg/kg tamoxifen by gavage were collected at day 4 post tamoxifen injection and were sectioned and stained with anti-phospho-RIPK3 antibody (1.35 μg/mL). Scale bar, 100 μm.

Potential solution

Depending on the quality of $\text{TNF}\alpha$, genetic background of your mice, and the animal housing environment, you may have to modify the amount of mouse $\text{TNF}\alpha$ injected, the time to harvest cecum samples, and the amount of anti-phospho-RIPK3 antibody used. The amount of $\text{TNF}\alpha$ for the induction of SIRS might vary from 200 μg/kg to 500 μg/kg.

Problem 5

Why can't I see any signals in other tissues?

Potential solution

First, you should confirm whether your sample tissues express RIPK3 or not. Signals of phospho-RIPK3 mostly appear in tissues that are damaged, so it is better that your sample tissues are visually damaged. In addition, when you do this staining, you should have a positive control (like the damaged ceca in TNF -induced SIRS mouse model) to exclude operational problems.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jiahuai Han (jhan@xmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101517>.

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AUTHOR CONTRIBUTIONS

L.L. and K.H. carried out most of the experiments with the help of C.R. and Y.Z.; Y.Z. and J.H. designed the experiments and interpreted the data; L.L., K.H., C.R., Y.Z., and J.H. wrote the manuscript; J.H. and Y.Z. conceived and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Cao, M., Chen, F., Xie, N., Cao, M.Y., Chen, P., Lou, Q., Zhao, Y., He, C., Zhang, S., Song, X., et al. (2018). c-Jun N-terminal kinases differentially regulate TNF- and TLRs-mediated necroptosis through their kinase-dependent and -independent activities. *Cell Death Dis.* 9, 1140. <https://doi.org/10.1038/s41419-018-1189-2>.
- Chen, W., Wu, J., Li, L., Zhang, Z., Ren, J., Liang, Y., Chen, F., Yang, C., Zhou, Z., Sean Su, S., et al. (2015). Ppm1b negatively regulates necroptosis through dephosphorylating Rip3. *Nat. Cell Biol.* 17, 434–444. <https://doi.org/10.1038/ncb3120>.
- Deng, B., Yang, D., Wu, H., Wang, L., Wu, R., Zhu, H., Huang, A., Song, J., Cai, T., Liu, S., et al. (2022). Ketamine inhibits TNF-alpha-induced cecal damage by enhancing RIP1 ubiquitination to attenuate lethal SIRS. *Cell Death Dis.* 8, 72. <https://doi.org/10.1038/s41420-022-00869-x>.
- He, P., Ai, T., Yang, Z.H., Wu, J., and Han, J. (2021). Detection of necroptosis by phospho-MLKL immunohistochemical labeling. *STAR Protoc.* 2, 100251. <https://doi.org/10.1016/j.xpro.2020.100251>.
- Wang, R., Li, H., Wu, J., Cai, Z.Y., Li, B., Ni, H., Qiu, X., Chen, H., Liu, W., Yang, Z.H., et al. (2020). Gut stem cell necroptosis by genome instability triggers bowel inflammation. *Nature* 580, 386–390. <https://doi.org/10.1038/s41586-020-2127-x>.
- Yang, Z.H., Wu, X.N., He, P., Wang, X., Wu, J., Ai, T., Zhong, C.Q., Wu, X., Cong, Y., Zhu, R., et al. (2020). A non-canonical PDK1-RSK signal diminishes procaspase-8-mediated necroptosis blockade. *Mol. Cell* 80, 296–310.e6. <https://doi.org/10.1016/j.molcel.2020.09.004>.
- Zhang, Y., Huang, K., Zhang, Y., Han, T., Li, L., Ruan, C., Sun, Y.H., Shi, W., Han, W., Wu, S.Q., et al. (2021). A unique death pathway keeps RIPK1 D325A mutant mice in check at embryonic day 10.5. *PLoS Biol.* 19, e3001304. <https://doi.org/10.1371/journal.pbio.3001304>.