Examining MLKL phosphorylation to detect necroptosis in murine mammary tumors

Necroptosis occurs predominantly in the center of late-stage tumors and necrototic cells are dispersed and difficult to be detected by Western blotting of key markers without enrichment by microdissection. To overcome these obstacles, this protocol provides a detailed immunohistochemistry-oriented approach including the steps of tumor isolation from mouse mammary tumor models, necrotic region identification by H&E staining, and necroptosis detection through examining mixed lineage kinase domain-like protein (MLKL) phosphorylation. This protocol could be applied to other types of solid tumors.

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

Examining MLKL phosphorylation to detect necroptosis in murine mammary tumors

Jin Young Baik,1,2,3 Peixing Wan,1,2 and Zheng-Gang Liu1,4,*

1Laboratory of Immune Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
2These authors contributed equally
3Technical contact
4Lead contact
*Correspondence: zgliu@helix.nih.gov
https://doi.org/10.1016/j.xpro.2022.101457

SUMMARY
Necroptosis occurs predominantly in the center of late-stage tumors and necrotic cells are dispersed and difficult to be detected by Western blotting of key markers without enrichment by microdissection. To overcome these obstacles, this protocol provides a detailed immunohistochemistry-oriented approach including the steps of tumor isolation from mouse mammary tumor models, necrotic region identification by H&E staining, and necroptosis detection through examining mixed lineage kinase domain-like protein (MLKL) phosphorylation. This protocol could be applied to other types of solid tumors. For complete details on the use and execution of this protocol, please refer to Baik et al. (2021).

BEFORE YOU BEGIN
Tumor necrosis commonly happens under hypoxic and nutrient deficient conditions in the advanced solid tumors (Jiao et al., 2018), and is closely related with poor prognosis of cancer patients. We recently reported that necroptosis, a programmed necrosis, plays a pivotal role in tumor necrosis and promotes tumor metastasis (Baik et al., 2021). Since tumor necrosis mainly happens in the core areas of solid tumors, identification of the necrotic regions before examining cell death with IHC is critical for necroptosis detection in solid tumors. This protocol guides you step-by-step from tumor model preparation and tumor isolation, identifying necrotic areas with H&E staining, to the IHC labeling of MLKL phosphorylation.

Although this protocol specifically focuses on murine mammary tumor, it can be easily adapted for necroptosis detection in other types of solid tumors.

Institutional permissions
The animal protocols of MVT-1 and MMTV-PyMT animal models are approved by NCI (IACUC no. CCBB-029).

MVT-1 orthotopic tumor model

© Timing: 5 weeks

1. Female 6–8 weeks old FVB/J mice are purchased from The Jackson Laboratory. All mice are housed in specific pathogen free (SPF) condition.
2. Preparation of the MVT-1 cells.
a. MVT-1 cells, which are originally derived from mammary tumor in MMTV-Myc-VEGF bitransgenic mouse (Pei et al., 2004) are cultured in DMEM.

b. 2 × 10^6 of MVT-1 cells are suspended in 100 μL of Matrigel Matrix solution (diluted 1:1 with 1× DPBS).

3. Injection of the MVT-1 cells.

a. The 100 μL of Matrigel Matrix solution containing MVT-1 cells are injected into the mammary fat pad of FVB/J mice (Figure 1A).

**MMTV-PyMT tumor model**

© Timing: 15 weeks

Female 5–6 weeks old FVB/N-Tg (MMTV-PyVT) 634 Mul/J mice are purchased from The Jackson Laboratory. All mice are housed in specific pathogen free (SPF) condition.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-phospho-MLKL (mouse specific) antibody (1:1000 dilution) | Abcam | ab196436 |
| Anti-cleaved caspase-3 (cl. casp-3) antibody (1:1000 dilution) | Cell Signal Technology | 9664 |
| Matrigel Matrix     | Corning | 354234 |
| 30% hydrogen peroxide (H₂O₂) solution | Sigma | H1009 |
| 10× Antigen retrieval solution (citrate pH 6.0) | Dako | S1699 |
| 1× DPBS (pH 7.4)    | KD medical | RGF-3200 |
| Sodium bicarbonate  | Mallinckrodt | 7412 |
| Permount (Mounting medium) | Fisher | SP15-100 |
| Methanol            | Fisher chemical | A411-4 |
| Ethanol             | Fisher chemical | A407P-4 |
| Xylene              | Lab Alley | XYL-1GAL |
| Hematoxylin         | Vector Laboratories | H-3404 |
| 4% buffered formalin (Z-fix) | ANATECH LTD | 170 |

**Critical commercial assays**

| VECTASTIN ABC Elite kit, Peroxidase (Rabbit IgG) (containing biotinylated goat anti-rabbit IgG) | Vector Laboratories | PK-6101 |
| ImmPACT® DAB Substrate, Peroxidase (HRP) | Vector Laboratories | SK-4105 |
| TUNEL Assay kit | Abcam | ab206386 |

**Experimental models: Cell lines**

| MVT-1 cells | Described in (Jiao et al., 2018) | MVT-1 cells |

**Experimental models: organisms/strains**

| FVB/J: female 6–8 weeks old | The Jackson Laboratory | 001800 |
| MMTV-PyMT: female 5–6 weeks old FVB/N-Tg (MMTV-PyVT) 634 Mul/J mice | The Jackson Laboratory | 002374 |

**Software and algorithms**

| ZEN software | ZEISS | N/A |

**Other**

| Miltex Eye Dressing Forceps, 4" Half Curved, Serrated | Miltex | V918-782 |
| Miltex Straight 4.5 in. Iris Scissors | Miltex | V95-304 |
| Scintillation vial | Electron Microscopy Sciences | 72632-FT |
| SLIP-RITE Cover Glass | Thermo Scientific | 102250 |
| IHC slide staining holder | N/A | N/A |

(Continued on next page)
**MATERIALS AND EQUIPMENT**

| REAGENT or RESOURCE SOURCE IDENTIFIER |
|--------------------------------------|
| IHC slide staining tray (black) with lid | N/A N/A |
| Manual IHC staining dish with lid | N/A N/A |
| Super HT PAP Pen | Research Products International Corp. 195505 |
| Culture dish (10 cm and 6 well plate) | Coming 430167 and 353046 |
| Cork board | N/A N/A |
| 50 mL conical tube | Coming 430828 |
| 100 µm nylon cell strainer | Fisher 22-363-549 |
| Razor blade | N/A N/A |
| Shake’N’Bake Hybridization Oven | Boekel Model 136400 |
| Black & Decker steamer | Black & Decker N/A |
| PYREX Griffin beaker | Fisher scientific N/A |
| 37°C incubator | N/A N/A |
| –20°C freezer | N/A N/A |
| Swing bucket centrifuge | N/A N/A |
| Shaker | Benchmark BT300 |
| Traceable™ Digital Carbon Fiber Calipers | Fisher Scientific 15-077-957 |
| Thermometer | 611 S-3SC Cole-Parmer |

**1× Antigen retrieval solution**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 10× Antigen retrieval solution (pH 6.0) | 1× (10%) | 15 mL |
| H₂O (molecular biology grade) | N/A | 135 mL |
| Total | N/A | 150 mL |

Freshly prepare the 1× Antigen retrieval solution each time, do not store it.

**3% Hydrogen peroxide solution**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 30% Hydrogen peroxide (H₂O₂) solution | 3% | 20 µL |
| Prechilled methanol | N/A | 180 mL |
| Total | N/A | 200 mL |

Methanol should be prechilled in –20°C for 30 min before use. 3% Hydrogen peroxide solution can be stored in 4°C up to 1 week.

**Primary antibody dilution ratio chart**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Anti-phospho-MLKL (mouse specific) antibody | 1:1000 | N/A |
| Anti-cleaved caspase-3 (cl. casp-3) antibody | 1:1000 | N/A |

Freshly dilute the primary antibody using 1× blocking buffer each time, do not store it.

**CRITICAL:** Adjust the pH to 6.0 with 0.1 M HCl. Bring up the volume to 1 liter with H₂O.

**CRITICAL:** Anti-phospho-MLKL antibody dilution ratio varies from batch to batch. It can be used up to 1:5000 dilution.
### 1x blocking buffer (from VECTASTIN ABC Elite kit)

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| Normal Goat serum (1:150 dilution)                | 2%                  | 3 drops  |
| DPBS (pH 7.4)                                     | 1x                  | 10 mL    |
| **Total**                                         | N/A                 | 10 mL    |

1x blocking buffer should be stored in 4°C up to 1 week.

### Secondary antibody solution (biotinylated goat anti-rabbit IgG from VECTASTAIN ABC Elite kit)

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| Biotinylated secondary antibody (1:200 dilution)  | N/A                 | 5 µL     |
| 1x Blocking buffer                                | N/A                 | 1 mL     |
| **Total**                                         | N/A                 | 1 mL     |

Freshly dilute the secondary antibody using 1x blocking buffer each time, do not store it.

### 1x Avidin-Biotin Complex (ABC) solution (from VECTASTAIN ABC Elite kit)

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| A                                                 | N/A                 | 20 µL    |
| B                                                 | N/A                 | 20 µL    |
| 1x DPBS (pH 7.4)                                  | N/A                 | 1000 µL  |
| **Total**                                         | N/A                 | 1040 µL  |

Freshly prepare the 1x ABC solution and should be incubated in 22°C for 1 h before use.

### ImmPACT DAB Solution

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| ImmPACT DAB Reagent 1                             | N/A                 | 1 drop   |
| ImmPACT DAB Diluent                               | N/A                 | 1 mL     |
| **Total**                                         | N/A                 | 1 mL     |

Freshly prepare the ImmPACT DAB solution each time, do not store it.

### 1x DPBST

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| 10x DPBS                                          | 1x                  | 100 mL   |
| Tween 20                                          | 0.05%               | 0.5 mL   |
| H₂O (molecular biology grade)                     | N/A                 | 900 mL   |
| **Total**                                         | N/A                 | 1 L      |

1x DPBST can be stored in 22°C up to 6 months.

### 0.1% Sodium bicarbonate solution

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| Sodium bicarbonate                                | 1%                  | 1 g      |
| H₂O (molecular biology grade)                     | N/A                 | 1 L      |
| **Total**                                         | N/A                 | 1 L      |

0.1% Sodium bicarbonate solution can be stored in 22°C up to 6 months.

### 95% Ethanol solution

| Reagent                                           | Final concentration | Amount   |
|---------------------------------------------------|---------------------|----------|
| 100% Ethanol                                      | 95%                 | 950 mL   |
| H₂O (molecular biology grade)                     | N/A                 | 50 mL    |
| **Total**                                         | N/A                 | 1 L      |

95% Ethanol should be stored in 22°C up to 6 months.
STEP-BY-STEP METHOD DETAILS

Tumor isolation

© Timing: 20 min per mouse

© Timing: 20 min per mouse

Preparation before isolation:

1. Euthanize mice by carbon dioxide (CO2) inhalation.
2. Pin down mouse on its back using 25-gauge needles (Figures 1B and 2A). Spray the mouse with 70% Ethanol.

Orthotopic model: Tumors from MVT-1 tumor bearing FVB mice.

Note: Tumor size should be measured with digital caliper every week. Once a tumor volume reaches 1500 mm³, the tumor can be collected. For tumor measurements, W is the tumor width (mm), L is the tumor length, and a tumor volume (V) is calculated using formula, \( V = \frac{L(W \times W)}{2} \).

---

70% Ethanol solution

| Reagent                     | Final concentration | Amount  |
|-----------------------------|---------------------|---------|
| 100% Ethanol                | 70%                 | 700 mL  |
| H2O (molecular biology grade)| N/A                 | 300 mL  |
| Total                       | N/A                 | 1 L     |

70% Ethanol should be stored in 22°C up to 6 months.

---

Figure 1. MVT-1 model and tumor isolation

(A) MVT-1 cells are injected into fat pad of no.2 mammary gland in FVB mouse.
(B) Tumor isolation. Lines indicate the incision area.
This step describes how the tumors are collected from both FVB mice bearing MVT-1 tumor and MMTV-PyMT mice.

3. Cut open the mouse skin from the area near the bottom of rib cage to neck with scissors (as shown in Figure 1B).
4. Gently hold the tumor capsule with half-curved forceps and dissect out the MVT-1 tumor.

△ CRITICAL: All the surgical tools should be autoclaved before use.

Genetically engineered transgenic mouse (GEMM) model: Tumors from MMTV-PyMT mice.

5. Cut open the skin from the area of the urethral orifice to the neck with scissors (Figure 2B).
6. Pin down the skin as shown below (Figure 2C) for an easy access to the tumors.
7. Gently hold the tumor capsule with half-curved forceps and dissect out the tumor.

△ CRITICAL: Although MMTV-PyMT mice have multiple tumors, MMTV-PyMT tumors are only collected from mammary glands no.3 and no.4 of each mouse as shown in the Figure 2C. The tumors from other mammary glands are not collected (Plante et al., 2011).

Tumor sample fixation

⏱ Timing: 5 min
8. Trim off any visible contamination of yellowish fat tissue or brachial lymph node from tumor samples.
9. Immediately put the tumor into a 10 cm culture dish and rinse once with cold sterile phosphate buffered saline (DPBS, pH 7.4).
10. The ball-shaped mammary tumor can be bisected along the longest diameter using a sharp razor.

**Note:** The longest diameter needs to reach 15 mm.

11. Transfer one half of each tumor sample into scintillation vials containing 10 mL of 4% buffered formalin (Z-fix) for histology.

**Note:** The other halves of tumors will be used for isolating primary tumor cells or Western blot assay.

12. After fixing tumor samples with Z-fix for 72 h at 22°C, rinse them once with 1× DPBS (pH 7.4). Dehydrate the fixed tumors and embed them in paraffin.

---

**Paraffin-embedding and H&E staining**

© Timing: 2 h for staining

Because necroptosis is a major cause of tumor necrosis (Jiao et al., 2018), which normally happens in the core areas of solid tumors, identification of the necrotic region before necroptosis detection is critical. Therefore, serial sectioning in tumor samples should be used for identifying the necrotic region. Then those samples with tumor necrosis can be used in evaluating necroptosis by phospho-MLKL IHC labeling.

The formalin-fixed tumor tissue is entrusted to Histoserv company for paraffin embedding and H&E staining following a general protocol (https://www.histoservinc.com/). The brief procedures of H&E staining (Fischer et al., 2008) are provided below.

13. Trim the top of the paraffin-embedded tumor block and cut 5 slides by 5 μm-serial-sectioning.

**Note:** H&E staining is performed on the 3rd slide from serial sections (Figure 3). The unstained slides will be used for IHC.

14. Place an unstained tumor slide in IHC slide staining holder. Deparaffinize the slide with 100% Xylene for 2 min.
15. Repeat step 2.
16. Hydrate tumor slide in 100% Ethanol for 2 min.
17. Repeat step 4.
18. Hydrate tumor slide once with 95% Ethanol for 2 min.
19. Wash tumor slide once with ddH₂O for 2 min.
20. Stain tumor slide with Carazzi’s hematoxylin for 3 min.
21. Wash tumor slide once with tap water and place the slide in 95% Ethanol before proceeding to the next step.
22. Counterstain tumor slide with eosin-phloxine for 30 s.
23. Dehydrate slide once with 95% Ethanol for 1 min and twice with 100% Ethanol for 1 min.
24. Rinse twice with 100% Xylene for 2 min.
25. Mount tumor slide with 200 μL of Permount solution dropwise to cover tumor tissue, followed by applying a coverslip.
Deparaffinization for IHC

**Timing:** ~40 min

The necrotic areas of tumors can be identified based on the unique morphology of tumor necrosis from H&E-stained tumor samples as shown in expected outcomes. After tumor samples with necrosis regions are identified by H&E staining, the serial sectioned slides of these samples will be stained with anti-p-MLKL antibody to examine necroptosis.

This step describes how to dissolve the paraffins from tumor slides for IHC.

26. Place tumor slides in slide staining holder (Figure 4C) and incubate at 56°C for 30 min in Shake’N’Bake hybridization oven.
27. Rinse three times with 100% of Xylene for 5 min: dip tumor slide holder in staining dish (Figure 4B) containing 150 mL of 100% Xylene. Move the slide holder up and down for a couple of times.
28. Rinse two times with 100% of Ethanol for 2 min: dip tumor slide holder in staining dish (Figure 4B) containing 150 mL of 100% Ethanol. Move the slide holder up and down after 1 min.
29. Rinse two times with 95% of Ethanol for 2 min: dip tumor slide holder in staining dish (Figure 4B) containing 150 mL of 95% Ethanol. Move the slide holder up and down after 1 min.
30. Rinse two times with 70% of Ethanol for 2 min: dip tumor slide holder in staining dish (Figure 4B) containing 150 mL of 70% Ethanol. Move the slide holder up and down after 1 min.

△ CRITICAL: All the deparaffinization steps are performed in a chemical hood. All the solution should be freshly made before use.

31. Wash tumor slides in ddH₂O for 5 min.

**Note:** All wash steps are performed on a shaker at 0.005 × g (50 rpm) at 22°C.
Antigen retrieval

**Timing:** ~60 min

This step describes the antigen retrieval method specific for p-MLKL antibody. Antigen retrieval is critical for the paraffin-embedded tissue section, which unmasks antigens to improve the binding of p-MLKL antibody.

32. Take out tumor slide from the slide holder and transfer into a 250 mL beaker (Figure 5B) containing 150 mL of 1× antigen retrieval solution.

*Note:* Steamer should be preheated to 95°C before use. Check the temperature with thermometer before proceeding to the next step.

33. Steam (Figures 5C and 5D) tumor slide for 10 min at 95°C.
34. Take out the beaker from the steamer and let slide cool down to 22°C.

*Note:* It will take about 45 min to cool down.

35. Take out tumor slide from the beaker and place it in a slide holder.
36. Wash tumor slides in 200 mL of ddH₂O for 5 min.

Blocking non-specific binding of antibody

**Timing:** ~90 min

This step describes how to reduce the endogenous peroxidase activity, which is critical for eliminating the background staining in tumor samples.

37. Transfer slide holder into a staining dish (Figure 4B) containing 200 mL of 3% hydrogen peroxide (H₂O₂) for 10 min at 22°C.
38. Transfer slide holder into a staining dish (Figure 4B) containing 200 mL of ddH2O and wash for 5 min.
39. Take out one slide each time and remove the residual ddH2O with a clean paper towel, then place it on a IHC slide staining tray (Figure 6).
40. Draw a circle around the tumor tissue with Super PAP Pen.
41. Add 100 μL of 1 x blocking buffer dropwise to the tumor tissue.
42. Close the tray lid and incubate for 60 min at 22°C on bench.

Primary antibody incubation

© Timing: ~16 h

This step describes how to incubate the primary antibody.

43. Remove the blocking buffer by tapping the slide edge on a clean paper towel.
44. Place tumor slide on a IHC slide staining tray. Add 1:1000 diluted p-MLKL antibody dropwise to the tumor tissues.

Note: The primary antibody solution should be freshly prepared before use.

45. Close the tray lid and incubate at 4°C for 16 h.

Secondary antibody incubation

© Timing: 90 min

This step describes how to incubate the secondary antibody.

46. Take out tumor slide from the IHC slide staining tray and place them in slide holder.
47. Wash tumor slide in 200 mL of 1 x DPBST for 10 min, repeat the wash step for three times.
48. Remove the excessive DPBST by tapping the slide edge on a clean paper towel.
49. Place tumor slide on a IHC slide staining tray and add 100 μL of secondary antibody solution dropwise to the tumor tissues.
50. Close the tray lid and incubate for 60 min at 22°C on bench.

*Note:* During the incubation time, prepare ABC solution and equilibrate to 22°C.

### ABC solution incubation

**Ο** Timing: 90 min

This step describes how antigen-antibody complexes will be detected by avidin and biotinylated enzyme (ABC solution).

1. Take out tumor slide from the IHC slide staining tray and place them in a slide holder.
2. Wash tumor slide in 200 mL of 1× DPBST for 10 min, repeat the wash step for three times.
3. Place tumor slide on a IHC slide staining tray and add 100 μL of ABC solution dropwise to the tumor tissues.
4. Close the tray lid and incubate for 60 min at 22°C on bench.

### Develop IHC staining signal

**Ο** Timing: ~40 min

This step describes DAB staining in tumor slides. The DAB HRP substrate will produce a dark brown reaction against avidin and biotinylated antigen-antibody complexes.

1. Take out tumor slide from the IHC slide staining tray and place them in slide holder.
2. Wash tumor slides in 200 mL of 1× DPBST for 10 min, repeat the wash step for three times.
3. Take out one slide each time and remove the residual 1× DPBST by tapping the slide edge on a clean paper towel.
4. Add 100 μL of DAB solution dropwise to the tumor tissue and incubate for up to 5 min at 22°C.

*Note:* DAB solution should be freshly prepared before use.

5. Once visible coloring is observed, immediately put the slide in fresh ddH₂O to stop the reaction.
The DAB solution should be washed out immediately after coloring to inhibit background staining (Figure 7). If there is no visible color change within 5 min, then increase the incubation time for up to 10 min. See troubleshooting.

Counter staining with hematoxylin

© Timing: ~40 min

This step describes hematoxylin staining in tumor slides. The hematoxylin stains cell nuclei with a purplish blue.

60. Place tumor slide in slide holder and wash them in 200 mL of ddH2O for 10 min, repeat the wash step twice.
61. Take out tumor slide from the slide holder and remove the residual ddH2O.
62. Place one tumor slide each time on bench top and add two drops of hematoxylin solution directly to tumor tissue and incubate for 4 min at 22°C.
63. Immediately put the slide in fresh ddH2O to stop the reaction.
64. Place tumor slide in slide holder and wash them in ddH2O for 5 min.
65. Take out slide holder with tumor tissues and dip in 200 mL of 0.1% sodium bicarbonate solution. 
   Move slides up and down for three times to ensure evenly bluing on slides.

Note: The bluing step should be less than 1 min to avoid over staining.

66. Put slide in holder and dip into 200 mL of ddH2O to stop the bluing reaction.
67. Transfer slide into 200 mL of fresh ddH2O and wash for 5 min.

Dehydration and mounting

© Timing: 30 min for dehydration

This step describes dehydration and mounting of tumor cells.

68. Rinse two times with 70% of Ethanol: put slide holder in staining dish (Figure 4B) containing 150 mL of 70% Ethanol for 2 min.
69. Rinse two times with 95% of Ethanol: put slide holder in staining dish (Figure 4B) containing 150 mL of 95% Ethanol for 2 min.
70. Rinse two times with 100% of Ethanol: put slide holder in staining dish (Figure 4B) containing 150 mL of 95% Ethanol for 2 min.
71. Rinse three times with 100% of Xylene: put slide holder in staining dish (Figure 4B) containing 150 mL of 100% Xylene for 5 min. Move the slide holder up and down for a couple of times during wash.
72. Take out one tumor slide each time and place it on bench top.
73. Remove the excessive Xylene solution and add 200 μL of Permount solution dropwise to cover the tumor tissue. Slowly apply the coverslip without any air bubbles.
74. Dry the tumor slide in a chemical hood for 16 h.

Note: All dehydration procedures should be performed in a chemical hood.

EXPECTED OUTCOMES
Representative H&E-stained tumor slides are shown in Figures 8 and 9. In the MVT-1 orthotopic tumors (1500 mm³), the necrotic areas are commonly observed in all of 8–12 slides sectioned between upper third and the lower third of a tumor block (Baik et al., 2021). Necroptotic tumor cells are characterized by organelles swelling, collapse, and deficiency of nuclear chromatin as dark-hematoxylin-stained cells (Figure 8, left panel). These features of necroptosis are quite distinct from the blebbing, nuclear fragmentation and chromatin condensation in apoptosis (Christofferson and Yuan, 2010); (Galluzzi and
Kroemer, 2008); (Galluzzi et al., 2018). Typical apoptotic cell death is observed in MVT-1 ZBP1 knock out tumor by H&E staining (Figure 8, right panel). A typical necrotic area with a mixture of viable cells, cells undergoing necroptosis, and necrotic cell debris is outlined with yellow dashed lines in Figure 9.

Although tumor necroptosis features are quite distinct from the apoptosis in H&E staining, it should be confirmed by IHC labeling of p-MLKL, a hallmark of necroptosis. As shown in Figure 9, tumor cell necroptosis is located surrounding the core of necrosis (no. 2 of Figure 9), with positive p-MLKL signal (no. 4 of Figure 9). Viable tumor cells with the negative staining of p-MLKL were also presented in Figure 9 (no. 1 and 3). P-MLKL positive necroptotic tumor cells are also detected surrounding the core of necrosis in the MMTV-PyMT tumor (Figure 10).

This feature of p-MLKL staining in tumor samples is unique and critical for the detection of necroptosis in tumors. These data demonstrated that identifying the necrotic areas with H&E staining in serial tumor sections is an important prerequisite for an ideal IHC staining-oriented necroptosis detection with anti-p-MLKL antibody in solid tumors.

LIMITATIONS

Quality of the p-MLKL antibody
Successful necroptosis detection is highly dependent on the quality of the p-MLKL antibody. The dilution ratio varies from batch to batch. A pilot study is recommended with titrations from 1:1000 to 1:10,000 for each lot of primary antibodies. However, a dilution of the antibody lower than 1:1000 is not recommended because of background staining.

Absence of RIPK3 or ZBP1 expression in tumor samples
Reports showed that RIPK3 or ZBP1, key molecules of necroptosis pathway, was often epigenetically silenced (e.g., DNA methylation) in tumors (Koo et al., 2015). Therefore, necroptosis could not happen in these RIPK3 or ZBP1-silenced tumor samples (Baik et al., 2021). If no necroptosis is detected by p-MLKL IHC in tumor samples, examining the expression levels of RIPK3 and ZBP1 in tumor lysates by Western blotting may be helpful in troubleshooting.
TROUBLESHOOTING

Problem 1
Fail to identify a necrotic area in tumor samples.

Potential solution
Tumor necroptosis only happens in solid tumors at their advanced stages when tumor cells are under nutrient-deficient stresses, particularly glucose deprivation (Baik et al., 2021). Early-stage solid tumors normally do not undergo necroptosis. As shown in Figure 10, in MMTV-PyMT tumors, the necrosis areas are gradually enlarged along with the tumor size and growth time (Jiao et al., 2018). The p-MLKL positive necroptotic cells are also gradually increased in these samples (Figure 10, bottom two panels). Therefore, it is important to conduct a time course (or size-dependent) collection of tumor samples, if possible, for H&E assay, and this will help to get an idea of necrosis development.

Problem 2
pH of the antigen retrieval buffer.

Potential solution
pH of the antigen retrieval buffer is a critical factor in p-MLKL IHC. Although the commercial 10× antigen retrieval buffer (Dako) supposed to be pH 6.0, an obvious pH increase was often noticed upon dilution to working solution. Therefore, it is highly recommended to routinely check the pH of the 1× antigen retrieval buffer before use. If only weak p-MLKL signal can be detected with the commercial antigen retrieval solution, it would be helpful to make fresh antigen retrieval buffer in house.

△ CRITICAL: Adjust the pH to 6.0 with 0.1 M HCl. Bring up the volume to 1 liter with H2O.

Problem 3
Lack of positive control for p-MLKL IHC staining.

Potential solution
To have a positive control of p-MLKL IHC staining, primary tumor cells can be isolated from tumors and are then cultured under glucose deprivation (GD) condition to induce necroptosis as shown in the recent study (Baik et al., 2021). If primary tumor cells are not available, it is recommended to epigenetically reprogram MVT-1 cells with 5AD before challenging them with GD. These cells can be fixed and embedded in paraffin to serve as the positive control for p-MLKL IHC.

Expected outcomes: Primary tumor cells isolated from MVT-1 or MMTV-PyMT tumors are highly responsive to GD. As presented in Figure 11, GD triggered the induction of p-MLKL in cultured MVT-1 and MMTV-PyMT primary tumor cells. Also, as shown in Figure 12, p-MLKL IHC staining is achieved in 5AD reprogrammed MVT-1 cells after GD treatment.

Problem 4
Classification of necroptotic vs apoptotic cell death in tumor samples.

| Reagent                                | Final concentration |
|----------------------------------------|---------------------|
| Sodium citrate                         | 10 mM               |
| Tween 20                               | 0.05%               |
| H2O (molecular biology grade)          | N/A                 |
| Total                                  | 1 L                 |
Potential solution

Although necroptosis may be the main cause of tumor necrosis, other forms of cell death, e.g., apoptosis could also occur in tumors, particularly when necroptosis is defective (Baik et al., 2021). It is highly recommended to examine apoptosis by TUNEL assay or cleaved caspase-3 (cl. casp-3) IHC in tumor samples. For cl. casp-3 IHC, general IHC method can be followed with the use of cl. casp-3 antibody with 1:1000 dilution.

Figure 11. p-MLKL is increased in MVT-1 or MMTV-PyMT primary tumor cells under GD condition

(A) Primary MVT-1 tumor cells were isolated from 5-week tumors of FVB/NJ mice implanted with MVT-1 cells. Isolated MVT-1 tumor cells were treated for the indicated time points with 0.5 mM glucose (GD) and examined by Western blotting.

(B) Primary MMTV-PyMT tumor cells from 15-week mice were isolated and treated for the indicated time points with GD then examined by Western blotting.

Material from: ‘Baik, J.Y, et al., ZBP1 not RIPK1 mediates tumor necroptosis in breast cancer, Nature Communications (2021) Nature Publishing Group [COPYRIGHT].

Figure 12. Example of a positive control for p-MLKL IHC staining

MVT-1 cells were pretreated with 5AD (upper panel), followed by GD for 16 h (middle panel) or 24 h (lower panel). Scale bar, 50 μm.
Expected outcomes: Here we used ZBP1 knock out tumors, where cell death switched from necroptosis to apoptosis, as an example. In Figure 13, tumor cell apoptosis was identified with positive cl. casp-3 (B) and TUNEL (C) signal.

Problem 5
Background staining.

Potential solution
The DAB solution incubation time is important to get a nice IHC image. If the background staining is too strong in tumor samples, there are two ways to fix it. First, figure out the optimal DAB incubation time as shown in Figure 7 (5 min incubation is ideal in this example). Second, reduce the secondary antibody incubation time. Incubation between 40 min to 60 min is ideal for murine mammary tumor.
Problem 6
Is necroptosis detection by p-MLKL IHC specific for tumor cells?

Potential solution
Solid tumors are composed of tumor cells and a mixture of other types of cells, including immune cells and fibroblasts. So, there is always the question whether other types of cells in tumors undergo necroptosis. In other words, are p-MLKL IHC detected necroptosis mainly from tumor cells? As presented in our previous study (Jiao et al., 2018), when the execution protein, MLKL, was depleted from xenograft tumor cells, the p-MLKL signal almost completely disappeared in tumor samples. Neither fibroblasts nor infiltrating immune cells from wildtype hosts have any obvious p-MLKL staining (Figure 14), which indicated that tumor cells is the major type of cells that underwent necroptosis in necrotic areas of solid tumors (Jiao et al., 2018).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zheng-gang Liu, zgliu@helix.nih.gov.

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate or analyze datasets.

ACKNOWLEDGMENTS
This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

AUTHOR CONTRIBUTIONS
J.Y.B. and P.W. prepared all the figures and drafted the manuscript. Z.-G.L. conceived, supervised, and directed the project and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Baik, J.Y., Liu, Z., Jiao, D., Kwon, H.J., Yan, J., Kadigamuwa, C., Choe, M., Lake, R., Kruhlak, M., Tandon, M., et al. (2021). ZBP1 not RIPK1 mediates tumor necroptosis in breast cancer. Nat. Commun. 12, 2666. https://doi.org/10.1038/s41467-021-23004-3.

Christofferson, D.E., and Yuan, J. (2010). Necroptosis as an alternative form of programmed cell death. Curr. Opin. Cell Biol. 22, 263–268. https://doi.org/10.1016/j.ceb.2009.12.003.

Fischer, A.H., Jacobson, K.A., Rose, J., and Zeller, R. (2008). Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. pdb.prot4996. https://doi.org/10.1101/pdb.prot4996.

Galluzzi, L., and Kroemer, G. (2008). Necroptosis: a specialized pathway of programmed necrosis. Cell 135, 1161–1163. https://doi.org/10.1016/j.cell.2008.12.004.

Galluzzi, L., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Agostinis, P., Alnemri, E.S., Altucci, L., Amelio, I., Andrews, D.W., et al. (2018). Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. Cell Death Differ. 25, 486–541. https://doi.org/10.1038/s41418-017-0012-4.

Jiao, D., Cai, Z., Choksi, S., Ma, D., Choe, M., Kwon, H.J., Baik, J.Y., Rowan, B.G., Liu, C., and Liu, Z.G. (2018). Necroptosis of tumor cells leads to tumor necrosis and promotes tumor metastasis. Cell Res. 28, 868–870. https://doi.org/10.1038/s41422-018-0088-y.

Koo, G.B., Morgan, M.J., Lee, D.G., Kim, W.J., Yoon, J.H., Koo, J.S., Kim, S.I., Kim, S.J., Son, M.K., Hong, S.S., et al. (2015). Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell Res. 25, 707–725. https://doi.org/10.1038/cr.2015.56.

Plante, I., Stewart, M.K.G., and Laird, D.W. (2011). Evaluation of mammary gland development and function in mouse models. J. Vis. Exp. https://doi.org/10.3791/2926.