Labeling of active proteases in fresh-frozen tissues by topical application of quenched activity-based probes

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Active enzymes, such as proteases, often serve as valuable biomarkers for various disease pathologies. Therefore, methods to detect specific enzyme activities in biological samples can provide information to guide disease detection and diagnosis and to increase our understanding of the biological roles of specific enzyme targets. In this protocol, we outline methods for the topical application of fluorescently quenched activity-based probes (qABPs) to fresh-frozen tissue samples. This technique enables rapid imaging of enzyme activity at cellular resolution, and it can be combined with antibody labeling for immunodiagnosis. In this method, fresh-frozen tissue sections are fixed, incubated with the probe and imaged using fluorescence microscopy. This provides an advance over classical immunohistochemistry (IHC) in that it is rapid (4–8 h) and inexpensive, and it provides information on enzyme activity. Furthermore, it can be used with any of the growing number of fluorescent ABPs to provide data for more effective disease monitoring and diagnosis.

INTRODUCTION

The ability to visualize the location and the activity levels of enzymes in relevant biological environments is a valuable way to begin to define their specific functions. In addition, many diverse enzymes become dysregulated in disease, and they therefore serve as biomarkers for diagnosis, detection and disease monitoring. Specifically, enzymes that serve as markers for cancer tissues have the potential to provide visual contrast to enhance efforts to surgically remove tumors. Surgeons strive to obtain negative margins during tumor resection, and their ability to completely remove a tumor is one of the critical factors that determines patient outcomes[1,2]. The presence of remaining tumor cells is associated with increased local recurrence, and it has been reported to indicate poor prognoses for patients with breast cancer, head and neck cancer, non-small-cell lung cancer and colorectal cancer[3–6]. Accurate assessment of tumor burden is therefore vital for successful surgical and patient outcomes.

A representative example of the current biopsy methodology used for the diagnosis and postoperative assessment of tumor margins occurs during lumpectomy procedures for surgical excision of breast cancer. One of the treatment options available for patients with breast cancer is breast-conserving surgery, in which the achievement of tumor-free surgical margins is desired to avoid local recurrence[7]. To aid surgeons in completely removing a tumor mass, pathologists currently rely on histopathology as the gold standard for determining surgical margin status. However, given the large quantities of breast tissue excised during lumpectomy procedures, it is not feasible to section and serially analyze the entire specimen in a timely manner. Current best-practice guidelines for histopathological sampling of breast tissue removed during surgery rely on a balance between cost-effectiveness and the likelihood of detecting lesions that change treatment plans[8]. Pathologists generally only sample the entire specimen if it fits into ≤20 cassettes for primary excisions, or ≤10 cassettes for re-excisions[8]. Therefore, it is not surprising that many patients require re-excision surgeries owing to positive surgical margins that are diagnosed postoperatively, with estimates ranging from 17 to 72% (refs. 9,10). A study assessing re-excision guideline recommendations based on sampling one or two sections per centimeter for grossly benign re-excision specimens found that sampling one section per centimeter resulted in the identification of only 81% of all lesions with clinical impact, whereas sampling two sections per centimeter successfully identified 95% of clinically impactful lesions[11]. High re-excision rates and limited histopathological sampling of many cancerous tissue types represent a substantial unmet clinical need for better margin assessment for both the surgeon and the pathologist.

Existing methods for visualization of tumor margins

When routine staining and morphology assessment are unable to provide comprehensive diagnostic answers, pathologists traditionally turn to advanced staining techniques such as IHC and *in situ* hybridization (ISH) to detect the presence of critical marker proteins and RNA or DNA sequences in tissue samples, respectively. Both techniques are commonly used for disease diagnosis, in which the presence or absence of particular proteins or sequences helps pathologists differentiate between disease states that look morphologically similar. Although these techniques are routine, they can be costly and time-consuming. Moreover, they only measure expression levels of a protein and provide no indication of its activity, which is often more accurately correlated with disease pathology than protein levels alone.

Several technological advances have been developed to improve the imaging of tumors and tumor margins, including MRI-enhanced surgery and fluorescence-guided surgery[12,13]. The use of fluorescently labeled markers that target tumors allows clear detection of margins, making it possible to achieve complete tumor resection while preserving healthy surrounding structures and tissue. Investigators have recently developed molecular imaging probes to image disease-associated proteases[14–18]. In addition to being viable targets for the treatment of disease, proteases are also potential biomarkers for diagnostic purposes[19]. Proteases have proven to be useful biomarkers for cancer because their increased

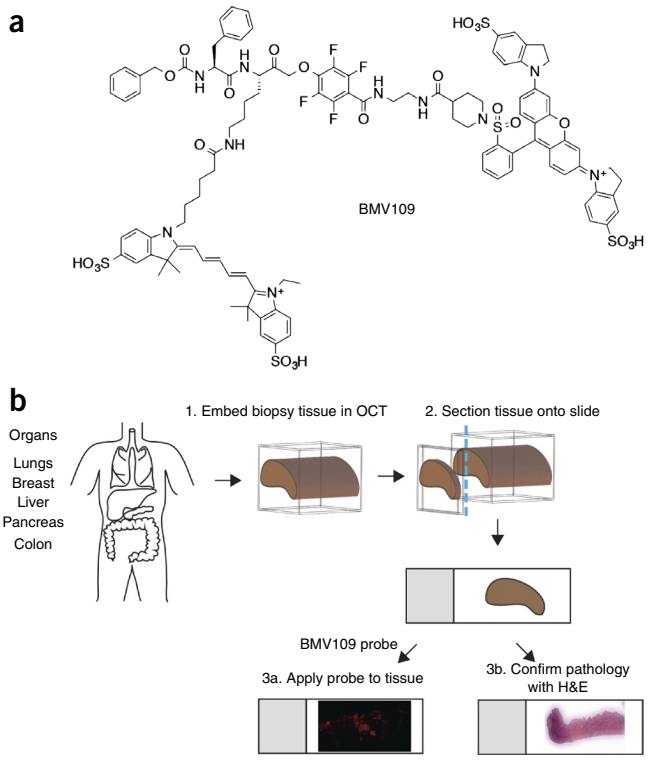

Figure 1 | Structure of BMV109 and general protocol for tissue labeling using a quenched activity-based probe. (**a**) Structure of the cathepsin probe BMV109. (**b**) Workflow diagram depicting the main steps for collecting biopsy specimens and the topical application of an activity-based probe to determine tumor margins and regions of disease burden from surrounding healthy tissue. OCT, optimum cutting temperature compound.

activity is linked with many of the hallmark processes of cancer, such as tissue remodeling, angiogenesis and cell death[20–22].

Basic research applications of qABPs

In addition to their potential use as markers of clinical disease, qABPs can be used in a similar manner in basic research applications to assess the presence of active enzymes in cells and tissues. As proteases serve as key marker proteins for a variety of physiological and pathological processes, such as tissue remodeling, apoptosis, fibrosis, cancer and inflammation[20–22], imaging active proteases with qABPs can be used to monitor disease progression, as well as to assess the efficacy of novel therapeutics targeting these diseases.

Cathepsins as targets for ABPs

The cysteine cathepsins are a family of 11 proteases that have diverse roles in both normal cellular physiology and in the pathology of various diseases. The cysteine cathepsins are normally localized to the lysosome, but they can be secreted extracellularly in a variety of physiological and pathological conditions[20]. Moreover, they are synthesized in an inactive preproenzyme form, and they are only activated under certain conditions, such as acidic pH. Thus, a direct measure of activity provides a more accurate correlation between the activation and localization of these proteases and the roles they have in disease pathology. The distinction between total protein levels of cysteine cathepsins measured using IHC and levels of active cysteine cathepsins measured with qABPs is highly valuable for understanding the basic biological functions of the cathepsins and for optimal diagnosis and disease monitoring.

Advantages and applications of using ABPs to assess tumor burden

In this protocol, we describe the use of qABPs targeting cysteine cathepsins to track protease activity in disease progression. ABPs have been used to detect aberrant protease activity in tissue samples. Our laboratory has developed multiple classes of qABPs that target cysteine cathepsins[14,18,23,24]. Most of these probes have been shown to be highly selective for specific cathepsin targets even when used *in vivo*. Specifically, the BMV109 probe used in this protocol labels multiple cysteine cathepsins, including B, L, S and X[18]. The benefit of using these covalent quenched probes over other

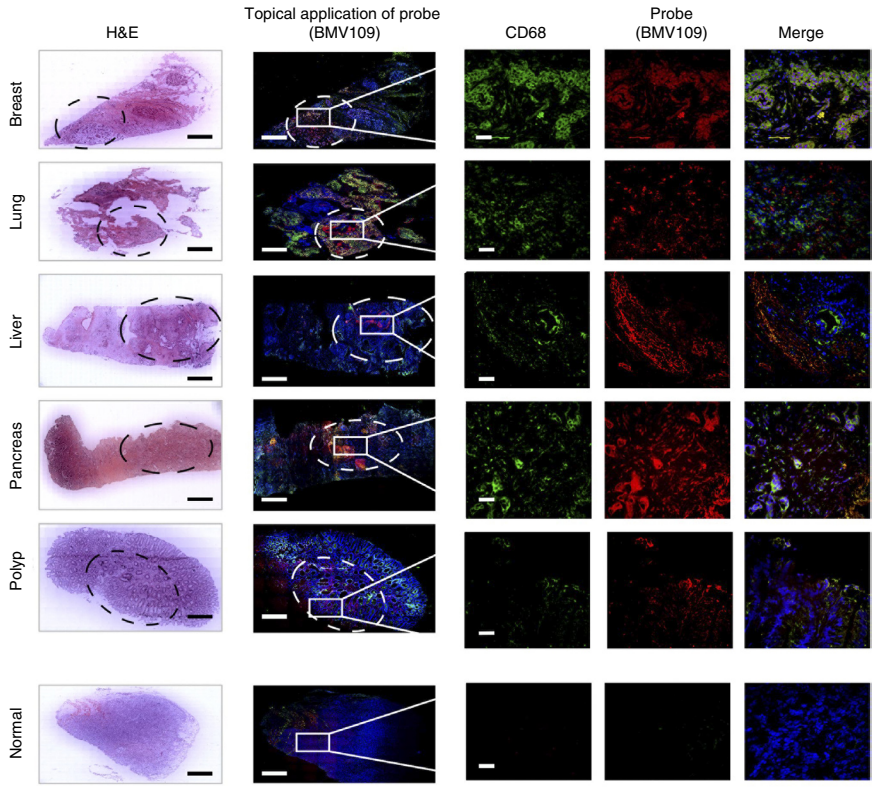

Figure 2 | Topical staining of fresh-frozen human biopsy tissues with the optical cathepsin probe BMV109. Frozen tissue sections from breast, lung, liver, pancreas and polyp cancer specimens along with a healthy tissue control were labeled with the optical probe BMV109 (red) and co-stained with the macrophage activation marker CD68 (green). Samples were tile-scanned at high resolution to generate full images. Insets show regions of interest (ROIs) at higher magnification. White scale bars on zoom images are 10 µm. Black and white scale bars on full tiled images are 1 mm. Paired H&E staining was performed to localize regions of disease. Black and white dashed lines show ROIs, and boxes show areas of magnification.

TABLE 1 | List of ABPs, their target proteases, and their sources of availability or references.

ABP	Target protease(s)	Source or reference
BMV109	Cysteine cathepsins	Verdoes *et al.*[18]
BODIPY-DCG-04	Cathepsins and calpains	Greenbaum *et al.*[31]
LE28	Legumain	Edgington *et al.*[32]
AWP28	Caspase-1	Puri *et al.*[33]
LE22	Caspase-6	Edgington *et al.*[34]
AB50-Cy5	Caspases: general	Edgington *et al.*[35]
YBN14	Cysteine cathepsins	Ben-Nun *et al.*[36]
Multiple	Caspases: general or specific to 1, 2, 3, 6, 8, 9, 10 or 13	ImmunoChemistry Technologies, Vergent Bioscience
DansylAhx$_3$L$_3$VS	Proteasome	Berkers *et al.*[37]
FP-Peg-TRM	Serine hydrolases	Patricelli *et al.*[38]
FP-fluorescein	Serine hydrolases	Liu *et al.*[39]
FP-Peg-fluorescein	Serine hydrolases	Patricelli *et al.*[38]

Table adapted from Bogyo *et al.*[40].

probes, such as substrate probes, is that they have higher protease selectivity, they rapidly penetrate cells, and they provide a durable signal owing to the fact that they remain bound to the active protease[25]. We have recently demonstrated that qABPs can be topically applied to colon tissues to detect intestinal cancer[17]. The probe rapidly and selectively labeled tumor cells, distinguishing cancerous tissue from surrounding healthy tissue with a positive predictive value of 90.32% (95% CI of 74.22 to 97.85%)[17]. This protocol details the original published method, and it includes additional data obtained using a specific inhibitor capable of blocking the probe signal in order to demonstrate the selectivity of the method for active cathepsins. The application and use of ABPs to identify novel biomarkers and regions of disease in biopsy tissues are also discussed. We present the use of qABPs as novel molecular imaging tools for the identification of tumor margins.

The continued development of fluorescent optical imaging devices and florescent probes will ultimately improve the intraoperative identification of tumors. The rapid nature of this method should allow integration into standard pathology workflows for clinical and basic research applications. Therefore, it could be not only implemented in the clinic for preoperative staging, but also optimized for intraoperative detection of cancers, thus providing surgeons real-time information to enhance surgical outcomes.

Experimental design

This protocol outlines the steps to visualize cysteine cathepsins as crucial marker proteins of inflammation and disease in fresh human biopsy samples by confocal microscopy using the qABP BMV109. BMV109 contains a Cy5 fluorophore, a sulfo-QSY21 quencher and a phenoxymethyl ketone electrophile that labels cysteine cathepsins B, S, L and X[17,18] (**Fig. 1a**). A schematic of the methodology is highlighted in **Figure 1b**. Human biopsy samples that contain pathological tissue have been successfully tested with our qABP BMV109 in lung, breast, liver, pancreas and colon (**Fig. 2**), although we anticipate successful detection of pathology in other diverse tissue samples. Although the use of BMV109 to target cysteine cathepsins is highlighted in this protocol, it is important to note that the general protocol can also be used with a variety of ABPs that target cathepsins and other proteases, such as the probes listed in **Table 1**. Because the synthesis of these probes is involved and requires substantial expertise, we have not outlined the synthesis methods but

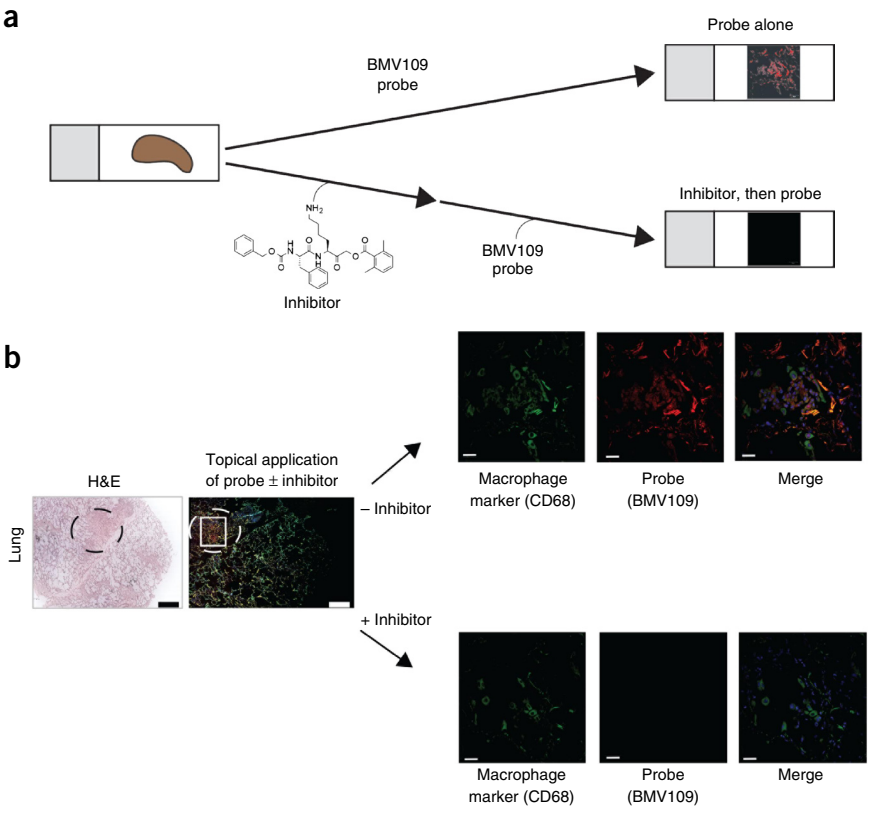

Figure 3 | Inhibition of probe signal by the addition of the cathepsin inhibitor GB111-NH$_2$ on fresh-frozen human biopsy tissues that have been labeled with the optical cathepsin probe BMV109. (**a**) Schematic representation detailing the labeling of serial sections with the BMV109 probe (top), or preincubating tissue sections with GB111-NH$_2$ inhibitor before labeling them with the probe (bottom). (**b**) Representative lung frozen tissue sections from a patient were labeled with and without the cathepsin inhibitor GB111-NH$_2$ for 1 h before labeling with the optical probe BMV109 (red) and co-staining with the macrophage activation marker CD68 (green). Samples were tile-scanned at high resolution to generate full images. Insets show ROIs at higher magnification. White scale bars on zoom images are 10 µm. Black and white scale bars on full tiled images are 1 mm. Paired H&E staining was performed to localize regions of disease. Black and white dashed lines show ROIs, and boxes show areas of magnification.

rather suggest directly contacting the laboratories that have published these reagents. We make all of our published ABPs available to researchers on request.

In this method, fresh biopsy specimens are taken from the patient and immediately embedded in optimum cutting temperature (OCT) embedding compound. OCT-embedded frozen tissues are sectioned onto slides. Tissues are briefly fixed in ice-cold acetone; this is a critical step in the preparation of histological sections to preserve biological tissues from decay and to prevent autolysis and putrefaction. The most common precipitating fixatives are ethanol and methanol; they are commonly used to fix frozen sections and smears, and they are less harsh on the tissue than formaldehyde. Acetone is also used, and it has been shown to produce better histological preservation[26–28]. Previous studies have shown that acetone fixation provides excellent preservation of both enzyme activity and cellular detail, making this method of fixation ideal for use with ABPs[29]. After fixation, tissues are incubated with the probe before imaging. For confirmation of pathological regions identified via fluorescence, serial sections are stained with H&E.

To confirm the specificity of the BMV109 probe for cysteine cathepsins, this methodology includes the preincubation of serial sections of biopsy tissue with a small-molecule inhibitor that targets the cysteine cathepsins, GB111-NH_2 (refs. 23,30). For this protocol, specimens are incubated with GB111-NH_2 before probe incubation, and they are then imaged to confirm inhibition of the probe signal associated with cathepsin activity (**Fig. 3**).

Limitations

The methodology described in this protocol requires preserved structure and function of target proteases for visualization. Therefore, only tissue samples that are fresh-frozen to preserve the activity of the proteins within the tissue can be successfully imaged using a qABP. Formalin-fixed and paraffin-embedded (FFPE) tissues are not suitable for this protocol.

MATERIALS

REAGENTS

- Optimum cutting temperature (OCT) embedding compound (Electron Microscopy Sciences Tissue-Tek, cat. no. 62550-01 (4583))
- Acetone (Fisher Scientific, CAS number 67-64-1)
- PBS, 1× without calcium and magnesium (Corning Cellgro Mediatech, ref. no. 21-040-CV)
- 3GM blocking Reagent (PerkinElmer, cat. no. FP1020)
- ABP for topical application, synthesized by our laboratory (others can be purchased) and stored in DMSO (Sigma Life Science, cat. no. D2438). Refer to **Table 1** for a list of commercially available and synthesized ABPs
  ! CAUTION DMSO can cause skin irritation. Wear appropriate personal protective equipment and manipulate it under a hood.
- ProLong Gold antifade mountant (Thermo Fisher Scientific, cat. no. P36934)
- Macrophage CD68 antibody (Bio-Rad, cat. no. 1815T)
- Secondary Alexa Fluor 488 antibody (Invitrogen, cat. no. A21151)
- DAPI (Life Technologies, cat. no. D13026)
- Water-immersion oil (Immersol W2010 Zeiss)

EQUIPMENT

- PAP pen (hydrophobic slide marker for staining procedures; RPI Research Products International)
- Vertical glass Coplin staining jar (Electron Microscopy Sciences Tissue-Tek, cat. no. 70315)
- Plastic slide-holder with cover for staining (Electron Microscopy Sciences Tissue-Tek, cat. no. 62541-01)
- Pipette tips, SS-L10 (Rainin, cat. no. 17005873)
- Pipette tips, SS-L250 (Rainin, cat. no. 17005875)
- Pipette tips, SS-L1000 (Rainin, cat. no. 17007090)
- Humidified chamber to incubate slides (an Eppendorf flipper rack filled with water and a flipper rack cover; Thermo Scientific. cat. no. 8760)
- Inverted confocal microscope (Zeiss LSM 700)
- Superfrost Plus microscope slides (Fisherbrand Fisher Scientific, Superfrost Plus, 25 × 75 mm, cat. no. 12-550-15, Thermo Fisher Scientific)
- Coverslips for slides (no. 1.5, Fisherbrand Fisher Scientific, 24 × 60 mm, cat. no. 22 266 882. Thermo Fisher Scientific) **▲ CRITICAL** It is important to use no. 1.5 thickness coverslips when imaging with the Zeiss LSM 700 microscope. The thickness of the coverslip is crucially important for high-resolution microscopy. Typical biological microscope objectives are designed for use with no. 1.5 coverslips.

PROCEDURE

Embedding and sectioning of human biopsy samples ● TIMING ~10 min

1| Embed freshly isolated human tissues in OCT.

2| Section the tissue into 6-µm sections on Superfrost Plus slides.

? TROUBLESHOOTING

■ PAUSE POINT Sectioned tissue can be used immediately for staining, or it can be stored at −80 °C indefinitely.

Staining protocol ● TIMING ~3.5–7 h

▲ CRITICAL If slides were stored at −80 °C, allow the frozen slides to completely thaw and air-dry for 20–30 min at room temperature (20–25 °C). This prevents sections from falling off the slides during antibody incubations. Place the slides in a slide rack that allows them to sit perpendicular to the bench top. Refer to **Figure 4a** for a pictorial representation.

3| Place slides in a vertical glass Coplin staining jar, with slides oriented perpendicular to the bench top at room temperature. Fix the tissue in cold acetone for 10 min by adding ~50–60 ml of ice-cold acetone (from a −20 °C freezer) to the Coplin jar. The liquid volume should entirely cover the glass portion of the slide, and the white label should stick up out of the liquid. Refer to **Figure 4b** for a pictorial representation.

? TROUBLESHOOTING

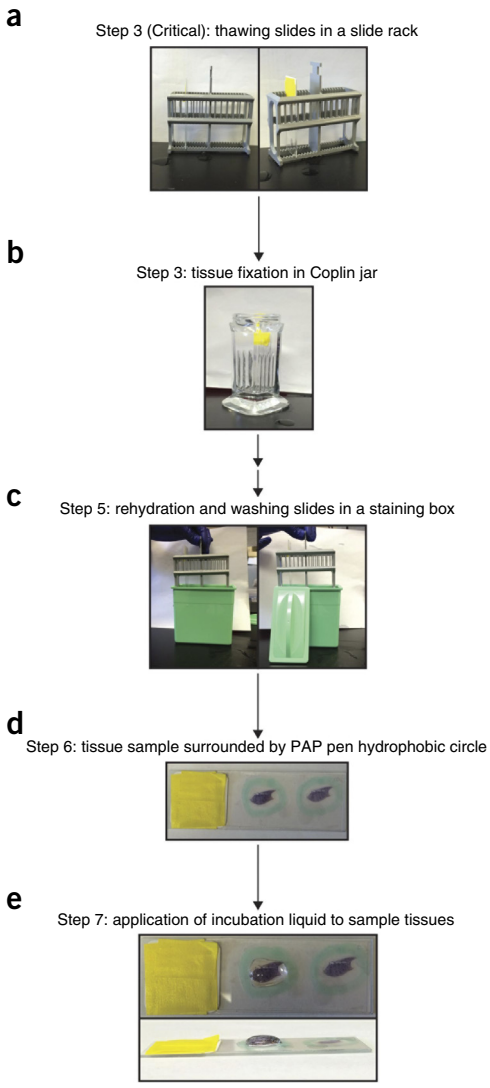

Figure 4 | Pictorial representation of protocol steps. (**a**) The appropriate orientation for slides thawing in the slide rack after removal from −80 °C. (**b**) Proper use of the glass Coplin jar to fix tissues in acetone. (**c**) Use of a staining box to rehydrate and wash samples. (**d**) Use of a PAP pen to draw a hydrophobic circle around tissue samples. (**e**) Vertical and horizontal views of the appropriate volume of liquid to use to cover the samples for incubation steps.

4| Remove the slides from the acetone and completely air-dry them for 20–30 min at room temperature. Slides should be placed in a slide rack that allows the slides to sit perpendicular to the bench top. Refer back to **Figure 4a** for a pictorial representation.

5| Place the slide rack containing the slides into a staining box in a perpendicular orientation to the bench top. Rehydrate tissues in PBS using three 5-min washes with gentle rocking by filling the staining box with ~250 ml of PBS. The entire glass portion of the slide should be covered with liquid. Change the PBS after each wash. Refer to **Figure 4c** for a pictorial representation.

6| Remove each slide one by one from the PBS, place them flat on an Eppendorf flipper rack, tissue side up, and draw a hydrophobic circle around each tissue section using a PAP pen. Refer to **Figure 4d** for a pictorial representation.

7| Block the sections for 1 h in premade PNB blocking buffer (0.5% (wt/vol) PNB powder dissolved in PBS). Depending on the size of the tissue section, 50–200 µl should be enough to cover each tissue section. Because of the cost and precious nature of both antibody and probe solutions, this step should be used to determine the minimum volume of liquid that will completely cover the tissue section. Use this volume for subsequent steps involving the incubation of tissue with the antibody and probe solutions. Refer to **Figure 4e** for a pictorial representation.

8| Remove the PNB buffer from slides, and then place the slides in a slide rack in a perpendicular orientation to the bench top. Place the rack in a staining box and wash the slides in PBS using three 5-min washes with gentle rocking by filling the staining box with ~250 ml of PBS such that the entire glass portion of the slide is covered with liquid. Change the PBS after each wash.

9| Remove excess moisture by gently tapping the slides, and place the slides flat on an Eppendorf flipper rack, tissue side up.

10| This step can be performed using the following options depending on whether the tissue section is being stained with probe alone (option A), probe plus an antibody of interest, e.g., CD68 (option B) or inhibitor GB111-NH_2 and probe (option C).

(A) Staining with probe alone

(i) Incubate the tissue with probe (1 µM in PBS) for 1 h at room temperature in the dark.

(B) Staining with probe plus an antibody of interest, e.g., CD68

(i) Place the slides flat with tissue side up on an Eppendorf flipper rack in which water has been filled in the holes to create a humidified chamber.

(ii) Apply the probe (1 µM) and primary antibody for CD68 in a 1:1,000 dilution in PNB buffer using the optimal volume to cover the tissue section, as determined in Step 7. Cover with the flipper rack cover, and incubate at room temperature in the dark for 1 h. If the quality of the antibody is not very good, or if it requires longer incubation, stain the section with the probe first, rinse the slides in PBS and then stain the sections with the primary antibody overnight in the humidified chamber at 4 °C.

(iii) Remove the primary antibody from the slides and place the slides in a slide rack and staining box in a perpendicular orientation to the bench top. Wash the slides by filling the staining box with ~250 ml of PBS such that the entire glass portion of the slide is covered with liquid. Perform three 5-min washes with gentle rocking, changing the PBS after each wash.

(iv) Place the slides flat with the tissue side up on an Eppendorf flipper rack, and incubate them with secondary antibody specific to CD68 (Alexa Fluor 488, Invitrogen, cat. no. A21151) using a 1:500 dilution in PNB buffer. Use the optimal volume to cover the tissue section, as determined in Step 7, for 1 h at room temperature.

(C) Control staining with inhibitor GB111-NH_2 before probe

(i) Incubate the tissue with 100 µM probe inhibitor (GB111-NH_2) to block cysteine cathepsin activity for 1 h at room temperature. Use the optimal volume to cover the tissue section, as determined in Step 7. Wash the slides and then incubate the tissue with the probe (1 µM in PBS) for 1 h at room temperature in the dark.

11| Place the slides in a slide rack and staining box in a perpendicular orientation to the bench top. Wash the slides by filling the staining box with ~250 ml of PBS, ensuring that the entire glass portion of the slide is covered with liquid. Perform three 5-min washes with gentle rocking. Change the PBS after each wash.
? TROUBLESHOOTING

12| Incubate with 2 µg/ml DAPI in PBS for 5 min using the optimal volume to cover the tissue section, as determined in Step 7.
▲ **CRITICAL STEP** Incubate with DAPI for exactly 5 min to prevent overstaining the cell nuclei.

13| Place the slides in a slide rack and staining box in a perpendicular orientation to the bench top. Wash the slides by filling the staining box with ~250 ml of PBS, ensuring that the entire glass portion of the slide is covered with liquid. Perform three 5-min washes with gentle rocking. Change the PBS after each wash.

14| Mount the sections in ProLong Gold antifade mounting medium by adding a few drops of the mounting medium to the sample and gently covering the specimen with a coverslip, avoiding the introduction of air bubbles.

15| Allow the slide to cure by laying it flat in the dark.
■ **PAUSE POINT** Slides can be imaged immediately or stored at 4 °C for ~1 month.

Imaging stained tissue sections ● TIMING 30–60 min per tissue section, depending on the size of the tissue
▲ **CRITICAL** Brief instructions are provided below for imaging tissue sections using a Zeiss LSM 700 confocal microscope. To image tissue sections live, use the 40× objective in the DAPI, Cy5 and FITC channels, and image tile scans using the 20× objective. For more detailed instructions, consult the Zeiss user manual.
16| Switch on the machine as instructed by the manufacturer.

17| Click on the 'Locate' tab in the Left Tool Area of the Zeiss LSM 700 software.

18| Place the specimen on the microscope stage. Make sure that the coverslip is facing down.

19| In the 'Objective list' box, select the required objective (40×). With the DAPI laser switched on, use the focusing drive to focus to the required specimen plane.

20| Click on the 'Acquisition' tab and then select the lasers of interest (in this experiment: DAPI, Cy5 for the probe and Alexa Fluor 488–FITC for the antibody of interest) and adjust the laser power and gain to achieve optimum signal for each channel.

21| *Setting up a Z-stack on a Zeiss LSM 700*. Begin by setting up a continuous *XY*-scan using the DAPI channel to focus on your specimen. Focus on the upper position of the specimen area using the focus drive of the microscope in order to determine where the *Z*-stack is to begin.

22| Click on the 'Set First' button to mark this point as the upper position of the *Z*-stack. Then, using the focus drive, move through the specimen and focus on the lower specimen area. Click on the 'Set Last' button.

23| Adjust the number of slices in the stack to match the optimal *Z*-interval for the particular stack size, objective lens and pinhole diameter used.

24| Finally, click on the 'Start Experiment' button to start the recording of the selected *Z*-stack.

25| *Tile scan acquisition on a Zeiss LSM 700*. The 'Tile Scan' function on the Zeiss allows for a frame to be created as an overview image of the specimen. The application of the 'Tile Scan' function requires an objective with a minimum magnification factor of 2.5×. Start by setting up a *Z*-stack on the thickest part of the specimen using the 20× objective.

26| Set up a tile scan. Use the bounding grid to select the area of the scan. Positions that should be part of the tile scan must be marked using 'Add'. By using these positions, a bounding grid is created, which defines the dimensions of the tile scan.

27| Select the channels that you want to scan (in this experiment; DAPI, Cy5 and Alexa Fluor 488–FITC).

28| Begin the experiment by clicking on the 'Start Experiment' button.

29| Save files as .lsm files and export images as .TIFF files, according to the manufacturer's instructions.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Specimen falls off the slide	Incorrect slides may have been used	Make sure to use Superfrost Plus microscope slides. These slides have good hydrophilic and adhesive properties. They will eliminate tissue loss during staining and save hours of slide preparation
3	Fixation artifacts, loss of signal, and/or increased nonspecific background signals	Overfixation with acetone	Ensure that the acetone used is cold (−20 °C), and make sure that the samples are not fixed for longer than 10 min
11	Nonspecific probe signal or high background	Insufficient washing of slides after the addition of the probe	The probes tend to be viscous and sticky; therefore, it is very important to rinse the slides thoroughly and for a long duration in large volumes of PBS with agitation

● TIMING

Steps 1 and 2, embedding and sectioning of human biopsy samples: ~10 min
Steps 3–15, staining protocol: ~3.5–7 h, depending on the optional use of antibodies for co-staining or control incubation with inhibitors before probe labeling
Steps 16–29, imaging stained tissue sections: ~30 min to 1 h per tissue section, depending on the size of the tissue

ANTICIPATED RESULTS

Figure 2 shows representative images expected from this protocol. Tile scans of whole biopsy specimens with heterogeneous sections of pathology and normal tissue show regions of enhanced signal in the red channel, corresponding to disease burden in serially sectioned H&E samples. Higher-magnification images of regions of enhanced signal show increased populations of macrophages. Merged images show colocalization of probe signal and macrophages. Results are shown for breast, lung, liver, pancreas and colon tissues. In contrast, normal tissue shows very little probe or macrophage signal.

Figure 3 details the application of the small-molecule inhibitor GB111-NH_2 to block probe signal as a measure of labeling specificity. The schematic in **Figure 3a** highlights using serial sections to incubate with probe alone, or preincubate with inhibitor before the probe is applied to the specimen. Representative images are shown in **Figure 3b**. In an area of pathology identified using the tile scan of lung tissue, higher-magnification imaging shows markedly reduced probe signal with preincubation of GB111-NH_2.

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AUTHOR CONTRIBUTIONS N.P.W. and M.G. designed and performed all experiments and drafted the manuscript. M.V. and L.O.O. designed and synthesized BMV109 and GB111-NH_2 used in experiments. E.S. helped establish the tile scans and obtain tissue samples for analysis. M.B. developed and coordinated the project, analyzed the data and prepared and edited the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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