Chapter 11

Live Cell Imaging and Profiling of Cysteine Cathepsin Activity Using a Quenched Activity-Based Probe

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Abstract

Since protease activity is highly regulated by structural and environmental influences, the abundance of a protease often does not directly correlate with its activity. Because in most of the cases it is the activity of a protease that gives rise to its biological relevance, tools to report on this activity are of great value to the research community. Activity-based probes (ABPs) are small molecule tools that allow for the monitoring and profiling of protease activities in complex biological systems. The class of fluorescent quenched ABPs (qABPs), being intrinsically "dark" and only emitting fluorescence after reaction with the target protease, are ideally suited for imaging techniques such as small animal noninvasive fluorescence imaging and live cell fluorescence microscopy. An additional powerful characteristic of qABPs is their covalent and irreversible modification of the labeled protease, enabling in-depth target characterization. Here we describe the synthesis of a pan-cysteine cathepsin qABP **BMV109** and the application of this probe to live cell fluorescence imaging and fluorescent SDS-PAGE cysteine cathepsin activity profiling.

Key words Activity-based probe, Protease, Cysteine cathepsin, Live cell imaging, Fluorescent SDS-PAGE

1 Introduction

Cysteine cathepsins are a family of proteases that govern many cellular processes, including antigen presentation, apoptosis, and tissue homeostasis [1]. Dysregulation of their proteolytic activity is a key step in the pathogenesis of cancer, atherosclerosis, and pancreatitis, among other inflammatory diseases [2–9]. Cysteine cathepsins are most abundantly expressed in the lysosome, where the acidic environment triggers their activation. In some circumstances, these proteases can be found in the nucleus, cytoplasm, or bound to the plasma membrane, and they can also be secreted [10–15]. In cancer, where hypoxia creates an acidic extracellular environment, secreted cathepsins are likely active and can mediate the migration and invasion of tumor cells through degradation of extracellular matrix components [16].

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The activity of cysteine cathepsins is regulated through numerous mechanisms. Zymogen activation occurs at low pH, where conformational changes make removal of the propeptide more energetically favorable [17]. Cleavage by upstream proteases such as legumain has also been implicated in their activation [18]. Several cysteine cathepsins are largely unstable and inactive at neutral pH (cathepsin S is still proteolytically active at neutral pH); however, binding to sulfated glycosaminoglycans (GAGs) such as heparin can have a stabilizing and activating effect [19, 20]. In other settings, GAGs can have inhibitory effects on proteolytic activity [21, 22]. Endogenous inhibitors, such as cystatins and some serpins, can also bind to cysteine cathepsins, competing with substrates for access to the active site [23]. Furthermore, cathepsin activity can be chemically modulated through introduction of exogenous inhibitors. Blocking the function of cathepsins with small-molecule inhibitors has been used as a therapeutic strategy in preclinical models of cancer and inflammation [3, 24].

Because cathepsins are highly regulated at the posttranslational level, total protein expression is rarely indicative of proteolytic activity. To directly monitor *active* enzymes, the field of activity-based proteomics has emerged [25–27]. Activity-based probes (ABPs) are small molecules containing an electrophilic moiety (warhead) that covalently binds to the active-site cysteine in an activity-dependent manner. Quenched ABPs (qABPs) for proteases contain a fluorophore–quencher pair [28, 29]. When an active protease performs a nucleophilic attack on the electrophile, the protease is covalently and irreversibly labeled and the quenching group is released, resulting in the emission of fluorescence (Fig. 1). The fact that qABPs are

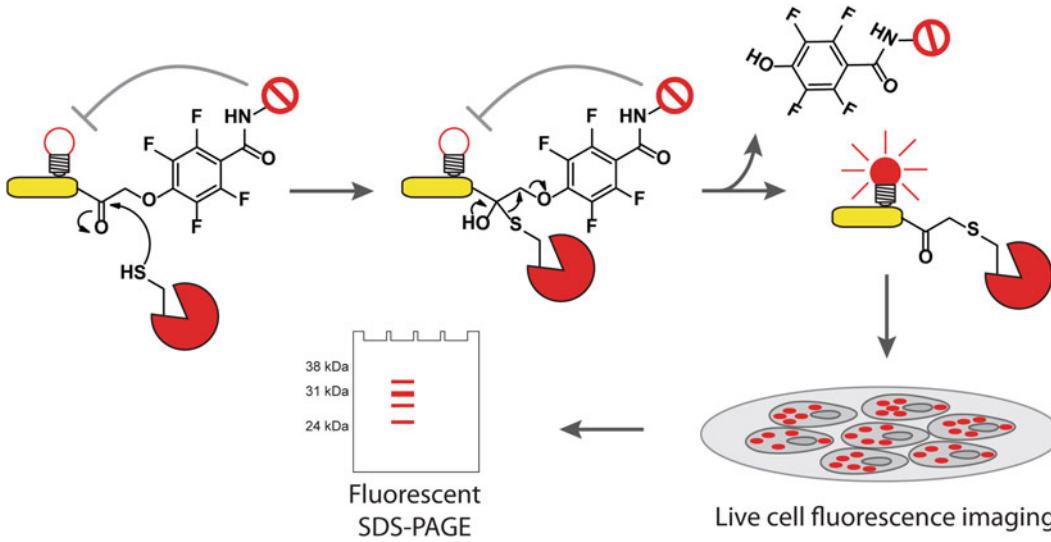


Fig. 1 Schematic presentation of the mechanism of action of quenched activity-based probes and their application in live cell fluorescence imaging and fluorescent SDS-PAGE

intrinsically "dark" (nonfluorescent) means that they are perfectly suited for live cell fluorescence confocal imaging. The use of qABPs enables fluorescence microscopy studies without the need for long-term probe washout periods or extensive washing after permeabilization of fixed cells, which is often required when using non-quenched ABPs. Because probe-binding is covalent and irreversible, labeled target proteases can be monitored by fluorescent SDS-PAGE. The proteins of probe-labeled samples are resolved by standard SDS-PAGE methods, after which the gel is scanned for fluorescence using a flat-bed laser scanner. This way fluorescence detected by microscopy can be linked to probe binding and unquenching by specific protease species.

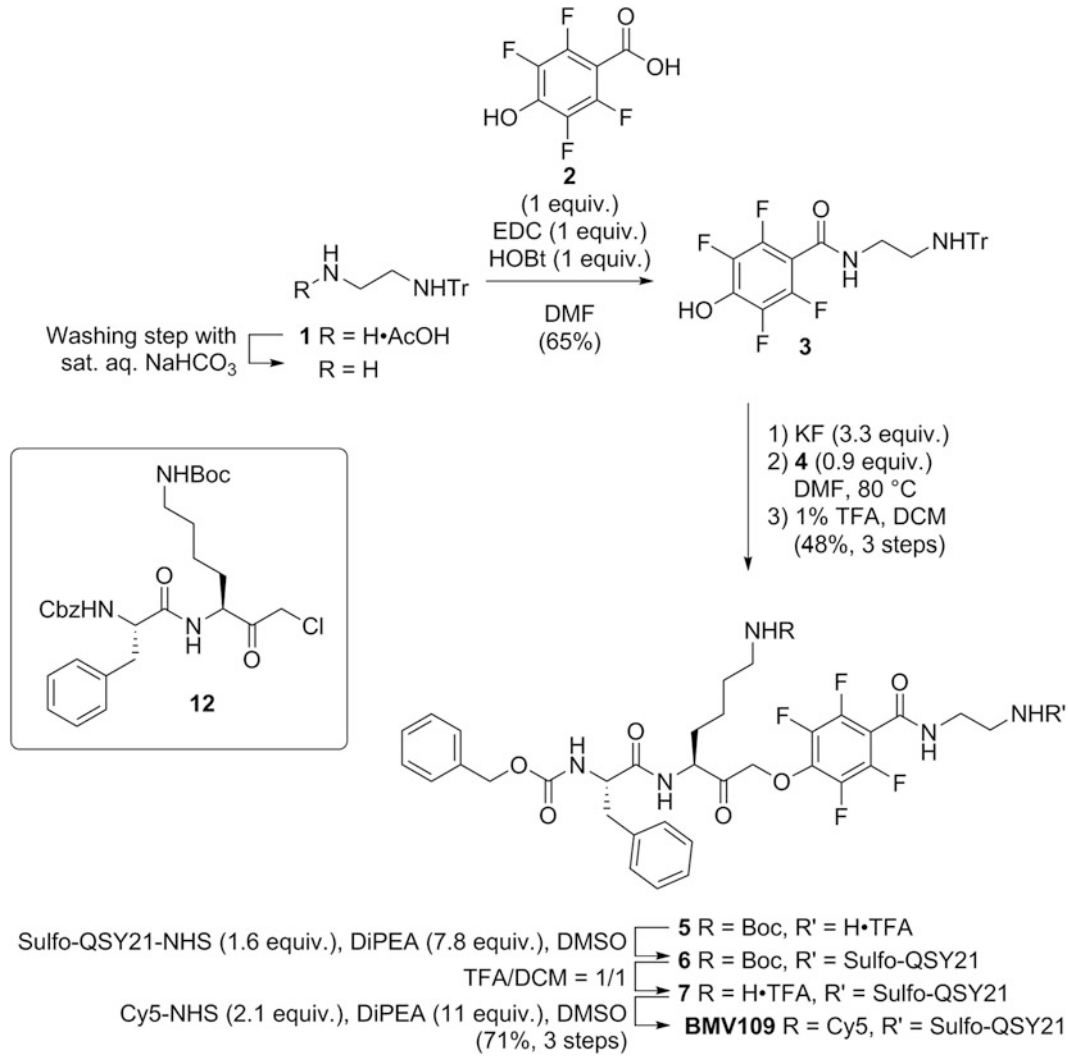
An optimized broad-spectrum qABP for cathepsins, **BMV109**, has recently been shown to have enhanced efficacy over previously described cathepsin probes [30]. **BMV109** has broad reactivity within the cysteine cathepsin family, allowing for simultaneous monitoring of the activities of cathepsins X, B, S, and L within the same experiment. This qABP also has improved solubility and yields brighter signal than other probes, most likely due to increased cathepsin reactivity of the phenoxymethyl ketone (PMK) warhead and enhanced cellular uptake. **BMV109** also demonstrated remarkable in vivo properties in a mouse model of breast cancer, yielding a tumor-specific signal that was 25 times brighter than the first-generation qABPs.

In this chapter, the synthesis of **BMV109** will be described (Subheadings 2.1 and 3.1) along with protocols for in vitro applications of the probe. The use of **BMV109** for live-cell fluorescence microscopy will be discussed (Subheadings 2.2 and 3.2), along with a protocol for fluorescent SDS-PAGE analysis of probe-labeled proteins (Subheadings 2.3 and 3.3). Finally, a method for validating the identity of probe-labeled cysteine cathepsin species by immunoprecipitation will be described (Subheadings 2.4 and 3.4). This experimental set up provides information about the cellular localization of the labeled pool of active cysteine cathepsins, which can directly be analyzed by fluorescent SDS-PAGE analysis and subsequently validated by immunoprecipitation (*see* **Note 1**).

2 Materials

2.1 Synthesis of BMV109

1. All solvents (dichloromethane [DCM], dimethylformamide [DMF], ethyl acetate, hexane, acetonitrile [CH_3CN], dimethylsulfoxide [DMSO], trifluoroacetic acid [TFA], toluene) were purchased from Fisher Scientific (HPLC grade).
2. Cbz-Phe-Lys(Boc)-CMK (chloromethyl ketone **4**, Scheme 1) was synthesized as previously reported (28) (*see* **Note 2**).
3. Sulfo-QSY21 *N*-hydroxysuccinimide (NHS) was synthesized as previously reported [31].



Scheme 1 Synthesis of the quenched activity-based probe **BMV109**

4. Sulfo-Cy5-NHS was synthesized in house, but can be purchased from a variety of vendors.
5. All other reagents (2,3,5,6-tetrafluoro-4-hydroxybenzoic acid, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride [EDC], potassium fluoride, triisopropylsilane, were purchased from Sigma Aldrich, except mono-trityl ethylenediamine acetic acid salt (Novabiochem) and HOBt monohydrate (Advanced Chemtech).
6. Saturated aqueous (sat. aq.) $NaHCO_3$ solution.
7. Anhydrous Na_2SO_4.
8. Reactions were analyzed by LC-MS using an API 150EX single-quadrupole mass spectrometer (Applied Biosystems).

9. Reverse-phase HPLC was conducted with an AKTA explorer 100 (Amersham Pharmacia Biotech) using C18 columns.
10. NMR spectra were recorded on a Varian 400 MHz (400/100), Varian 500 MHz (500/125) or a Varian Inova 600 MHz (600/150 MHz) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz.

2.2 Live Cell Imaging of Cysteine Cathepsin Activity and Lysosomal Staining

1. Nunc™ Lab-Tek™ II Chambered Coverglass, 4 Chamber (Thermo Scientific).
2. (Adherent) cell type of interest (here mouse bone marrow-derived dendritic cells are used (*see* **Note 3**).
3. Appropriate culture media (phenol red-free if available).
4. Humidified 37 °C incubator (5% CO2 in air).
5. Cysteine cathepsin inhibitor (here a 20 mM JPM-OEt solution in DMSO is used. *See* **Note 4**).
6. Cysteine cathepsin qABP: 5 mM **BMV109** solution in DMSO.
7. LysoTracker Green DND-26 (1 mM in DMSO, Invitrogen, L7526), 1 in 10 dilution (100 μM) in DMSO.
8. Inverted (confocal) fluorescence microscope (here an epi-fluorescence Leica DMI6000 microscope with a 63× oil 1.4 NA objective, a metal halide EL6000 lamp for excitation, a DFC365FX CCD camera [Leica] and GFP and Y5 filter sets [for lysotracker green and Cy5, respectively; all from Leica] was used).

2.3 Fluorescent SDS-PAGE Analysis

1. Refrigerated microcentrifuge.
2. Lysis buffer: 50 mM Citrate [pH 5.5], 5 mM DDT, 0.5% CHAPS, and 0.1% Triton X.
3. Ice bucket.
4. 4× sample buffer: 0.8 g SDS, 1 mL 2 M Tris pH 6.8, 4 mL glycerol, 0.4 mL 2-mercaptoethanol, 8 mg bromophenol blue, 4.6 mL water.
5. Eppendorf tube heating block at 95 °C.
6. SDS-PAGE gel 0.75 mm or 1.5 mm. 15% polyacrylamide running gel and 4% stacking gel.
7. Amersham ECL Plex Fluorescent Rainbow Markers (GE Healthcare).
8. Fluorescent flat-bed scanner (here the Typhoon Trio+, GE Healthcare was used).
9. Coomassie staining solution: for 1 L, add 100 mL of glacial acetic acid to 500 mL of distilled water. Add 400 mL of methanol and mix. Add 1 g of Coomassie R250 dye and mix. Filter to remove particulates.

10. Microwave.
11. Orbital shaker.
12. Destaining solution: for 1 L, add 100 mL of glacial acetic acid to 700 mL of distilled water. Add 200 mL of methanol and mix.
13. Flat-bed scanner.

2.4 Validation of Probe-Labeled Species by Immunoprecipitation

1. **BMV109**-labeled protein lysate (leftover from 3.3 or prepared fresh).
2. IP Buffer: PBS (pH 7.4), 0.5% Nonidet P-40 (NP-40; v,v), 1 mM EDTA.
3. Anti-Cathepsin Antibodies:

   Goat anti-mouse Cathepsin B (R&D, AF965).

   Goat anti-mouse Cathepsin X (R&D, AF1033).

   Goat anti-mouse Cathepsin L (R&D, AF1515).

   Goat anti-human Cathepsin S (Abcam, 18822).
4. Protein A/G agarose beads (Santa Cruz, sc-2003).
5. Nutator or rocker.
6. 2× Sample Buffer (dilute the 4× sample buffer prepared in Subheading 2.3 two times with distilled water).
7. 0.9% Sodium Chloride.
8. Insulin syringe.
9. Materials required for SDS-PAGE analysis (*see* Subheading 2.3).

3 Methods

3.1 Synthesis of BMV109

In this section, the synthesis of the cysteine cathepsin qABP **BMV109** is described (Scheme 1). All chemical reactions should be performed in a chemistry fume hood using laboratory glassware unless stated otherwise. All water-sensitive reactions are performed in anhydrous solvents under positive pressure of argon.

1. To remove the acetic acid, take up the mono-trityl ethylenediamine acetic acid salt (**1**) (100 mg, 0.28 mmol) in DCM and washed with sat. aq. $NaHCO_3$ and dry the organic phase over Na_2SO_4 and concentrate in vacuo.
2. Dissolve the amine in DMF (5 mL) and add HOBt monohydrate (43 mg, 0.28 mmol, 1 equiv.), EDC (54 mg, 0.28 mmol, 1 equiv.) and 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid (**2**) (59 mg, 0.28 mmol, 1 equiv.). Stir the reaction mixture overnight under argon atmosphere and concentrate in vacuo.
3. Isolate 2,3,5,6-tetrafluoro-4-hydroxy-*N*-(2-(tritylamino)ethyl) benzamide (**3**) by silica gel flash column chromatography puri-

fication (20%→35% ethyl acetate in hexane). Expected isolated yield: 0.18 mmol, 65% (*see* **Note 5**).

4. Suspend potassium fluoride (6.3 mg, 108 μmol, 3.3 equiv.) in DMF by sonication for 5 min. under argon atmosphere. Add phenol **3** (19.4 mg, 39 μmol) and stir the reaction mixture for 10 min.
5. Add chloromethyl ketone **4** (20 mg, 36 μmol, 0.9 equiv.) and stir the reaction mixture in an oil bath at 80 °C for 3 h. Concentrate the reaction mixture in vacuo.
6. Take up the crude product in 1% TFA in DCM (the solution will turn yellow) and stir for 30 min. Quench the carbocations (yellow color) by the addition of triisopropylsilane until the solution turns colorless. Add toluene to the reaction mixture and concentrate in vacuo.
7. Isolate intermediate **5** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 20:80–60:40 over 20 min; 5 mL/min) and lyophilize the product containing fractions. Expected isolated yield: 15.4 mg, 17.3 μmol, 48% from CMK **4**, as a white powder (*see* **Note 6**).
8. Dissolve intermediate **5** (5.8 mg, 6.5 μmol) in DMSO (100 μl) in a 1.5 mL eppendorf tube and add Sulfo-QSY21-NHS (9.75 mg, 10.39 μmol, 1.6 equiv.) and DiPEA (8.4 μl, 50.5 μmol, 7.8 equiv.). Vortex to dissolve all components and react overnight in the dark.
9. Isolate Sulfo-QSY21 amide-intermediate **6** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 25:75–55:45 over 20 min; 5 mL/min), followed by lyophilization of the product containing fractions to afford a dark blue powder.
10. Take up the Sulfo-QSY21 amide-intermediate **6** in a 50% TFA solution in DCM and react for 30 min. to remove the Boc protective group. Coevaporate the solvents with toluene (3×) and dissolve the residue (intermediate **7**) in DMSO (250 μl).
11. Add Sulfo-Cy5-NHS (10.5 mg, 13.9 μmol, 2.1 equiv.) and DiPEA (12 μl, 72 μmol, 11 equiv.) and after 4–20 h isolate **BMV109** by HPLC (preparatory reverse phase C_{18} column, CH_3CN/H_2O 0.1% TFA, 25:75–45:55 over 20 min; 5 mL/min), followed by lyophilization of the product containing fractions. Expected isolated yield: 7.74 mg, 4.61 μmol, 71% over three steps as a dark blue powder (*see* **Note 7**).

3.2 Live Cell Imaging of Cysteine Cathepsin Activity and Lysosomal Staining

This protocol can be used to image the localization of cysteine cathepsin activity within an adherent cell. When **BMV109** is added to the culture media, it freely enters the cell, largely by endocytosis. When reacting with active cysteine cathepsins, the probe is unquenched and fluorescence can be detected by fluorescence (confocal) microscopy. To ensure that the fluorescent signal is cathepsin-dependent, a control experiment should be performed

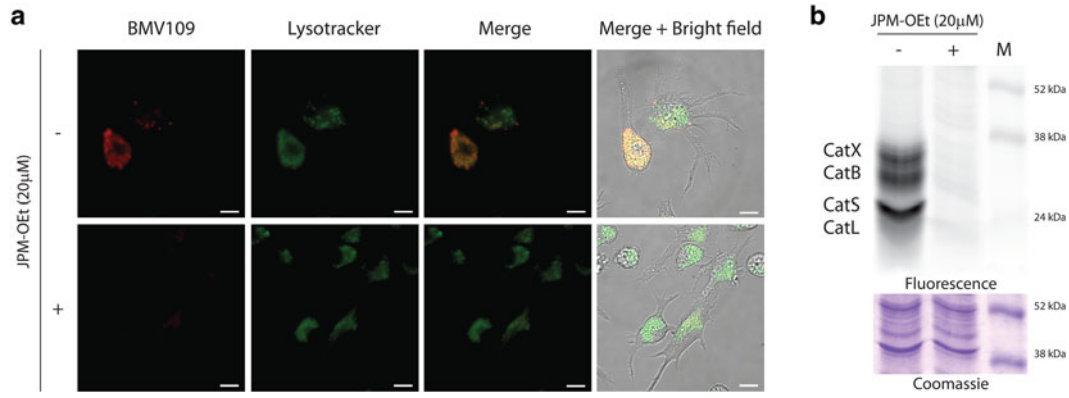


Fig. 2 Live cell fluorescence microscopy of **BMV109** labeled cells and fluorescent SDS-PAGE of the labeled protease targets. (**a**) Living mouse bone marrow-derived dendritic cells were labeled with 5 μM **BMV109** (*red*) and 100 nM Lysotracker (*green*) and imaged for probe fluorescence. In the cysteine cathepsin inhibitor (JPM-OEt) pretreated sample (lower panels) minimal **BMV109** fluorescence is observed. Scale bar represents 10 μm. (**b**) After imaging, the cells were harvested and lysed and the proteins were resolved on SDS-PAGE (15 %). The probe labeled protease bands were visualized using a fluorescent flat-bed scanner. The cysteine cathepsin inhibitor (JPM-OEt) pretreated sample (center lane) confirms **BMV109** target protease inhibition. Equal protein loading was confirmed by Coomassie blue staining (*lower panel*)

in parallel, in which a broad-spectrum cysteine cathepsin inhibitor is applied 30 min prior to **BMV109** addition. This inhibitor irreversibly inactivates the cysteine cathepsins, rendering them unable to unquench **BMV109**. As observed in Fig. 2a, inhibitor-treated cells have little fluorescent signal by microscopy compared to non-inhibited cells, which yield increasing fluorescence over time. In order to verify that the localization of the cathepsin signal is lysosomal, LysoTracker Green may be added. This dye is fluorescent specifically in acidic compartments of the cell. Co-localization of green and red signal indicates a lysosomal distribution of cathepsins, as shown in Fig. 2a. If signal in other organelles is detected (i.e., nucleus, plasma membrane), other cellular markers may be required. All steps of the following protocol need to be performed using sterile materials in a cell culture flow cabinet.

1. Harvest and count the cells of interest and resuspend them in culture media at a concentration of 150,000 cells/mL (*see* **Note 8**).
2. Seed the cells by gently pipetting 1 mL of the cell suspension in each well of the chambered coverglass. Culture the cells for 1–12 h to allow the cells to adhere (*see* **Note 9**).
3. For the inhibitor pretreatment control well, pipet 0.5 μl of the 20 mM JPM-OEt solution in DMSO in a sterile 1.5 mL eppendorf tube. Transfer 499 μl of conditioned culture media from the appropriate well to the eppendorf tube containing the inhibitor (final JPM-OEt concentration 20 μM).

4. Carefully aspirate off the remaining culture media from the well. Quickly to prevent the cells from running dry, pipet the inhibitor-containing conditioned culture media from the 1.5 mL eppendorf tube back onto the cells gently. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 30 min.
5. For the rest of the wells, repeat **steps 3** and **4** using 0.5 μl DMSO instead of 0.5 μl of the 20 mM JPM-OEt solution in DMSO.
6. After the 30 min. inhibitor (or DMSO) pre-incubation, pipet 0.5 μl of the 5 mM **BMV109** solution in DMSO in a sterile 1.5 mL eppendorp tube (*see* **Note 10**). Transfer the culture media from the well to the eppendorf tube containing the probe (final **BMV109** concentration 5 μM).
7. Gently pipet the probe-containing conditioned culture media back onto the cells. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 1 h (*see* **Note 10**).
8. After 1 h probe incubation time, pipet 0.5 μl of the 100 μM Lysotracker solution in DMSO in a sterile 1.5 mL eppendorf tube. Transfer the culture media from the well to the eppendorf tube containing the Lysotracker (final Lysotracker concentration 100 nM) (*see* **Note 11**).
9. Gently pipet the Lysotracker-containing conditioned culture media back onto the cells. Incubate at 37 °C and 5 % CO_2 in a humidified incubator for 1 h.
10. After 1 h of Lysotracker incubation (and 2 h of total probe incubation time), remove the Lysotracker containing media from the cells and replace it with pre-warmed fresh (phenol red-free) culture media.
11. Image the cells in each well for Lysotracker Green and Cy5 fluorescence on an inverted fluorescence (confocal) microscope (Fig. 2a).

3.3 Fluorescent SDS-PAGE Analysis

In this section, a biochemical analysis of the cells that were imaged in the 4-chambered coverglass wells (Subheading 3.2) is described. The cells are lysed and analyzed by fluorescent SDS-PAGE. The detected fluorescent bands correlate with the fluorescence that is observed by fluorescence microscopy, providing a biochemical readout of cysteine cathepsin activity. As observed in Fig. 2b, the JPM-OEt-treated inhibitor control yields dramatically decreased labeling intensity compared to the non-inhibited sample, which has several bands corresponding to the sizes of cathepsin X, B, S, and L. This labeling pattern may vary depending on the cell type and conditions used. To ensure that the lanes are loaded with equal protein amounts, a Coomassie stain may be performed.

This protocol can be applied to cells (or lysates) labeled with **BMV109** in any experimental setting. Treatments aimed to modulate cathepsin activity (i.e., cytokines such as IL-4, serum

deprivation, apoptotic agents, etc.) may be added to the cells prior to the addition of **BMV109**. A densitometry analysis can be performed on the fluorescent bands, allowing for a quantitative readout of relative cathepsin activities.

1. Carefully aspirate off culture media from the well and wash the cells by gently pipetting 1 mL of PBS onto the cells. Aspirate off the PBS and pipet 500 μl PBS into the well.
2. Remove the cells from the bottom of the well using the blunt end of a 200 μl pipet tip by placing the tip perpendicular to the bottom of the well and gently scraping in a circular fashion. Transfer the PBS cell suspension to a sterile 1.5 mL eppendorf tube and place on ice. Repeat **steps 1** and **2** for each well.
3. Centrifuge the cell suspension for 30 s at 9,500 × *g* and remove the supernatant. Resuspend the cell pellet in 9 μl lysis buffer and incubate the cells on ice for 15 min. Centrifuge at 4 °C for 20 min. at 21,000 × *g*.
4. Transfer the supernatants to new 1.5 mL eppendorf tubes and add 3 μl 4× sample buffer to each sample (*see* **Note 12**). Heat the samples to denature and reduce the proteins for 5 min. at 95 °C. Centrifuge the samples for 10 s. at full speed (*see* **Note 13**).
5. Load the samples and the fluorescent molecular weight marker (5 μl) on the SDS-PAGE gel and run the first 15 min. on 80 V to run the proteins through the stacking gel. Increase the voltage to 130 V and stop the gel when the dye front has run off (*see* **Note 14**).
6. Remove the gel from the glass and place it in a tray with water for transfer to the Typhoon Imager (*see* **Note 15**). Place the wet gel slab on the scanner surface (*see* **Note 16**) and select the area to be scanned with the software. Choose the excitation and emission parameters for the Cy5 dye and scan the gel. If the fluorescent signal is saturated, lower the PMT value and repeat the scan (*see* **Note 17**).
7. To control for equal protein loading per lane, Coomassie staining can be performed (*see* **Note 18**). After scanning, transfer the gel to a microwave proof tray. Add enough Coomassie staining solution to submerge the gel completely. Place the tray in the microwave and heat for 10–15 s (prevent boiling of the solution). Place the tray on the orbital shaker and shake for 5 min.
8. Dispose the staining solution and wash the gel with water. Add enough destaining solution to submerge the gel completely. Place the tray in the microwave and heat for 10–15 s (prevent boiling of the solution). Roll up a tissue (Kimwipes work well—the tissue will soak up the Coomassie), wet it with water and place it along the edge of the tray and shake until the gel has destained to satisfaction (*see* **Note 19**). Scan the gel on a flat-bed scanner.

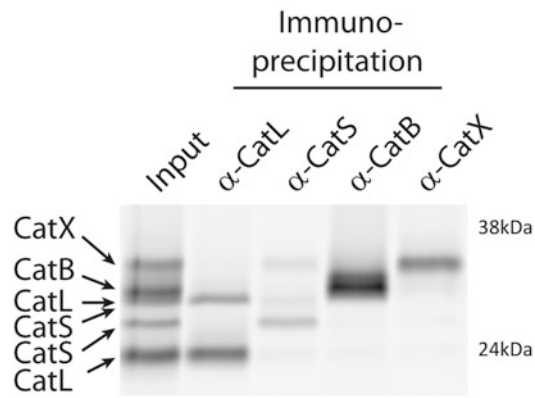


Fig. 3 Cysteine cathepsin immunoprecipitation. After labeling with **BMV109**, target cysteine cathepsin annotation can be validated by immunoprecipitation and analyzed by fluorescent SDS-PAGE

3.4 Validation of Probe-Labeled Species by Immunoprecipitation

Different cell types may express different forms of active cathepsins, and the identities of the labeled proteins may be difficult to determine based on size and banding pattern alone. To definitively assign each signal to the correct cathepsin family member, an immunoprecipitation experiment may be performed. This involves incubating probe-labeled lysate with cathepsin-specific antibodies in the presence of Protein A/G agarose beads. The precipitated proteins are then analyzed by fluorescent SDS-PAGE alongside an input sample. In the example shown (Fig. 3), lysates from RAW cell macrophages labeled with **BMV109** were immunoprecipitated with cathepsin-specific antibodies, and the labeled proteins were assigned to multiple species of cathepsins X, B, S, and L.

1. Prepare **BMV109**-labeled lysate according to Subheading 3.3. After boiling the lysate in sample buffer, set aside 30 μg total protein for the input sample. Then aliquot 100 μg protein into four microcentrifuge tubes for immunoprecipitation of the cathepsin X, B, S, and L.
2. To the four tubes, add 500 μl IP buffer and the indicated antibody: 10 μl for X, B, and L (2 μg) or 20 μl for S (4 μg) (*see* **Note 20**). Incubate the tubes on ice for 10 min.
3. Meanwhile, prepare the Protein A/G agarose beads. Aliquot 160 μl bead slurry (40 μl for each immunoprecipitation) into a microcentrifuge tube. Add 1.3 mL IP buffer to wash the beads and invert the tube once. Centrifuge for 30 s at high speed in a table-top centrifuge and then remove the buffer, taking care not to disturb the bead pellet. Add 160 μl of IP buffer, resuspend the slurry, and then aliquot 50 μl to each of the 4 immunoprecipitation tubes prepared in **step 2**. Nutate or rock the samples overnight at 4 °C.

4. The next morning, centrifuge for 30 s at high speed in a tabletop centrifuge and aspirate supernatant. Wash the beads four times with 1 mL IP buffer, and 1 time with 0.9% sodium chloride, centrifuging and removing the supernatant between each wash. After the final wash, remove all traces of liquid with an insulin syringe, and resuspend the beads with 20 μl 2× sample buffer. Boil for 10 min. and then load the immunoprecipitations along with the input sample on a 15% gel as described in Subheading 3.3. To analyze, scan the gel for fluorescence, also according to the method in Subheading 3.3.

4 Notes

1. For in vivo applications of **BMV109**, refer to [30].
2. **CAUTION!** The method involves the preparation and use of diazomethane. This is a **HIGHLY EXPLOSIVE** reagent! Special glassware and safety precautions are needed. If not experienced in performing these reactions, seek advice from a chemistry department. Diazomethane is prepared as described in the Aldrich technical Bulletin (AL-180), which is available online.
3. For the generation of bone marrow-derived dendritic cells *see* for example [32, 33].
4. E-64d or leupeptin are commonly used commercially available cysteine cathepsin inhibitors that could be used instead of JPM-OEt.
5. ^{1}H NMR (400 MHz, DMSO) δ = 8.77 (t, *J* = 6.0, 1H), 7.39 (d, *J* = 7.8, 6H), 7.27 (t, *J* = 7.7, 6H), 7.17 (t, *J* = 7.2, 3H), 3.40–3.35 (m, 2H), 2.86–2.77 (m, 1H), 2.14–2.04 (m, 2H).
6. ^{1}H NMR (400 MHz, CD_3OD) δ = 7.36–7.12 (m, 10H), 5.05 (s, 2H), 4.86–4.81 (m, 2H), 4.42–4.37 (m, 2H), 3.64 (t, *J* = 6.5, 2H), 3.14 (t, *J* = 6.5, 2H), 3.08 (dd, *J* = 13.9, 7.2, 1H), 2.99 (t, *J* = 6.5, 2H), 2.91 (dd, *J* = 13.9, 8.4, 1H), 1.90–1.78 (m, 1H), 1.62–1.48 (m, 1H), 1.41 (s, 9H), 1.46–1.20 (m, 4H).
   HRMS (ESI): calc. for $C_{38}H_{46}F_4N_5O_8^+$ 776.3277, found 776.3286, $C_{38}H_{45}F_4N_5O_8Na^+$ 798.3096, found 798.3104.
7. 7. ^{1}H NMR (600 MHz, CD_3CN) δ 8.12–8.08 (m, 1H), 8.01–7.93 (m, 2H), 7.89–7.85 (m, 2H), 7.75 (dd, *J* = 12.0, 1.5 Hz, 2H), 7.72 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.69 (dd, *J* = 8.3, 1.2 Hz, 1H), 7.66 (s, 2H), 7.62–7.57 (m, 2H), 7.51 (dd, *J* = 8.4, 5.1 Hz, 2H), 7.46 (d, *J* = 9.4 Hz, 2H), 7.41–7.35 (m, 3H), 7.24 (s, 1H), 7.22 (s, 1H), 7.21–7.14 (m, 6H), 7.13–7.09 (m, 6H), 7.05 (dd, *J* = 8.8, 4.6 Hz, 1H), 6.39 (t, *J* = 12.8 Hz, 1H), 6.11 (t, *J* = 12.6 Hz, 1H), 4.87 (q, *J* = 12.7 Hz, 2H), 4.83 (dd, *J* = 39.7, 14.1 Hz, 2H), 4.23–4.12 (m, 4H), 3.93 (q, *J* = 7.2 Hz, 2H), 3.86 (t, *J* = 7.4 Hz, 2H), 3.34 (dd, *J* = 6.7, 4.1 Hz, 2H), 3.28–3.15 (m, 9H), 3.04–2.92 (m, 3H), 2.80–2.74 (m, 1H), 2.45 (t, *J* = 11.9 Hz, 2H), 2.15–2.09 (m, 1H), 2.09–2.03 (m, 2H), 1.74–1.58 (m, 7H),

1.57 (s, 6H), 1.55 (s, 6H), 1.49 (dd, J=15.1, 7.4 Hz, 4H), 1.35–1.22 (m, 7H), 1.20 (t, J=7.3 Hz, 3H), 1.16–1.12 (m, 4H).

HRMS (ESI): calc. for $C_{107}H_{106}F_4N_{10}O_{23}S_5^{2-}$ 1067,2991, found 1067,2961, $C_{107}H_{107}F_4N_{10}O_{23}S_5^{-}$ 2135.6056, found 2135.5990, $C_{107}H_{106}F_4N_{10}O_{23}S_5Na^{-}$ 2157.5875, found 2157.5831.

8. The optimal amount of cells and seeding density needed for microscopy and subsequent fluorescent SDS-PAGE needs to be determined for every new cell type to be analyzed. The amount of active cathepsins differs per cell type.
9. Optimize this step for every new cell type to be analyzed.
10. Probe concentration and labeling time should be optimized to achieve the desired labeling saturation for every new cell type to be analyzed.
11. Lysotracker concentration and incubation time should be optimized for every new cell type to be analyzed.
12. In this example, the lysate from all the cells per well (equal cell numbers) is loaded on the SDS-PAGE gel. When using larger cell numbers the protein concentration of the lysate could be determined. Typically 30 μg of total protein is sufficient for detection of cathepsin labeling by fluorescent SDS-PAGE analysis, but this differs per cell type and should be optimized. Overloading of the gel will result in poor gel scans.
13. At this point the samples could be frozen for continuation at a later stage. Before the next step, heat the samples for 5 min at 95 °C, followed by centrifugation for 10 s. at full speed.
14. For a more detailed protocol on standard SDS-PAGE methods, *see* Ref. [34].
15. Depending on the plates being used, it may also be possible to scan the gel while still in the plates. This option ensures that the gel does not tear during handling and that it stays flat for the scan. In this case, choose "+3" for the focal plane. Otherwise, use the default "platen" option.
16. Place the gel on the Typhoon with the side of the gel (the short end) at the bottom to reduce scanning time (the Typhoon scans from left to right).
17. After saving the .GEL file it can be analyzed and saved as a .TIFF file using the open source image processing software ImageJ (http://imagej.net/).
18. Alternatively, the gel may be transferred to a membrane and blotted with cathepsin antibodies to evaluate total protein expression. Other proteins such as actin or GAPDH may be assessed as loading controls.
19. To speed up the process, the destaining solution can be replaced and **step 8** can be repeated.
20. If antibodies other than those recommended are used, the amount of antibody will need to be optimized.

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